IMPACTS OF ORGANIC AND CONVENTIONAL NEUROTOXIC PESTICIDES ON A PEST AND A POLLINATOR IN ALMOND AGROECOSYSTEMS (*PRUNUS DULCIS*)

BY

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THESIS

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ABSTRACT

The navel orangeworm *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) is an economic pest on a wide variety of tree fruit and nut crops, including almonds, pistachios, and walnuts in California orchards. Rising demand for these high-value cash crops has led to an increased reliance on synthetic insecticides to reduce damage from this pest. At the same time, consumer interest has risen for organic products, resulting in the development and usage of novel chemical controls, such as spinosyns, derived from natural products, in contrast with conventional pesticides, such as neonicotinoids, synthesized based on natural products. Both spinosyn and neonicotinoid insecticides act on binding sites of the nicotinic acetylcholine receptors. Although used against specific target pests, both spinosyns and neonicotinoids are broad-spectrum insecticides and thus can have non-target effects on beneficial arthropods in agroecosystems, including pollinators. In this study, focused on the almond production system in California's Central Valley, I examined impacts of the synthetic neonicotinoids and the organic spinosyns on the pest navel orangeworm and the managed pollinator *Apis mellifera*.

Chapter 1 describes an examination of the toxicity and mode of detoxification of two neonicotinoids and a spinosyn in navel orangeworm utilizing inhibitors of specific enzyme groups to test for synergistic enhancement of toxicity. The effects of the cytochrome P450 monooxygenase (P450) inhibitor piperonyl butoxide and the glutathione-S-transferase inhibitor diethyl maleate on the toxicity of the insecticides acetamiprid, clothianidin, and spinosad (a natural fermentation product made up of a mixture of Spinosyn A and D) were assessed in a series of bioassays with first instar *A. transitella* larvae from a laboratory strain (CPQ). An increase in the toxicity of acetamiprid and spinosad by piperonyl butoxide implicates P450s in the detoxification of at least some representatives of neonicotinoid and spinosyn insecticide

classes. However, there were no significant differences in toxicity of the neonicotinoid clothianidin with the addition of either piperonyl butoxide or diethyl maleate. Because P450-mediated resistance to the pyrethroid insecticide bifenthrin has been reported in Kern County, California, determining the range of pesticides metabolized by P450s is critical for making management decisions designed to delay widespread cross- and multiple-resistance acquisition.

In Chapter 2, I measured the behavioral responses of free-flying honey bees (*Apis mellifera*) to the presence of pesticides in a sugar water solution, including neonicotinoids currently or previously used in almond orchards for management of navel orangeworm or other pests. Sugar water feeders containing imidacloprid at 5 ng/mL were more attractive to free-flying honey bees relative to unamended sugar water feeders. No attraction or repellency was seen with acetamiprid at concentrations of 50 ng/mL, 500 ng/mL, and 5 µg/mL. Thus, in semi-field conditions, honey bees may be attracted to and preferentially feed on foods contaminated with a highly toxic pesticide at low concentrations.

My findings highlight the need to consider unintended and non-target impacts of using insecticides to control pests in crop settings, such as almond orchards. Growers that use pesticide rotation to avoid resistance or utilize organic products need to consider if there is any overlap in detoxification enzymes used by their target pest and what impacts residues of these different insecticides might have on foraging honey bees on which they rely to pollinate their crop.

ACKNOWLEDGMENTS

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Lastly, thank you to my husband, Michael Jared Thomas, for his patience, help, and support through the long, hot days at the Pollinatarium, ridiculously late timepoints, and odd hours I kept through all this work.

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I. Detoxification modes of neonicotinoid and spinosyn pesticides by the navel orangeworm, *Amyelois transitella*[1]

Introduction

The navel orangeworm *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) is an economic pest on a wide variety of tree fruit and nut crops, including almonds, pistachios, and walnuts in California orchards (Bentley 2008, Wade 1961, Zalom et al. 2012). In almond orchards, adult females lay their eggs on unharvested nuts ("mummies") on the tree, on nuts on the ground at the end of the season, and on crop nuts after hull split has occurred, in general preferring injured or diseased fruits (Heinrich 1956). Neonates tunnel into the nut, consume the nutmeat, and cause direct damage by producing frass and webbing, and feed internally until pupation (Curtis and Barnes 1977). This damage facilitates infection by *Aspergillus* fungi, which produce aflatoxins (Campbell et al. 2003, Molyneux et al. 2007, Zalom et al. 2012, Palumbo et al. 2014); contamination of these high-value crops by these mycotoxins causes millions of dollars in losses each year (Campbell et al. 2003, Molyneux et al. 2007, van Egmond et al. 2007, Palumbo et al. 2014). Minimizing damage is a priority because of the high value of tree nuts. Currently, a combination of sanitation (removal of unharvested fruits) and insecticides is used for control of this insect in almonds and pistachios (Higbee and Siegel 2009).

Almond acreage has increased from 428,000 acres in 1996 to 870,000 acres in 2014 (USDA NASS 2014) to keep up with rising demand for this high-value cash crop, which in turn has resulted in an increased reliance on synthetic organic insecticides to reduce damage due to this pest. At the same time, consumer interest in organically grown almonds has led to the

[1] Portions of the data, figures, and analysis represented in this chapter appeared in the Journal of Economic Entomology: Demkovich, M., Dana, C.E., Siegel, J.P., and M.R. Berenbaum. 2015. Effect of piperonyl butoxide on the toxicity of four classes of insecticides to navel orangeworm (*Amyelois transitella*) (Lepidoptera: Pyralidae). Journal of Economic Entomology 108: 2753-2760. Figures are reprinted with permission of the publisher.

development of novel control chemicals. Both conventional and organic pesticides, especially if considered broad-spectrum, can have nontarget effects on beneficial arthropods in agroecosystems, including pollinators. Moreover, use of pesticides for management of pests other than navel orangeworm can lead to inadvertent exposure of this insect to additional classes of pesticides, increasing selection for cross- or multiple resistance.

Insecticides registered for use in California orchards include members of the pyrethroid (IRAC 3A), organophosphate (IRAC 1B), diamide (IRAC 28) diacylhydrazine (IRAC 18), neonicotinoid (IRAC 4A), and spinosyn (IRAC 5) classes. Pyrethroids are the most widely used insecticides in navel orangeworm management; in 2014, pyrethroids were two of the top five most frequently applied insecticides in almond orchards and four of the top five most frequently applied insecticides in pistachio orchards (CDPR 2014). Continued use of the broad-spectrum pyrethroids is in question; bifenthrin resistance has already been reported (Demkovich et al. 2015b) and concerns have been raised about its use in the spring due to the risks it presents to natural enemies at this time of year (Zalom et al. 2001, Hamby et al. 2013). Reduced risk pesticides, including spinosyns, such as spinosad and spinetoram, have been examined as alternatives to the broad-spectrum pyrethroids. Spinosyns are produced as a natural fermentation product from a soil actinomycete and thus are approved for use in organic agriculture (Cleveland et. al 2002).

Little is known, however, about how navel orangeworm detoxifies these novel, reduced-risk pesticides. Cytochrome P450s have been implicated in navel orangeworm detoxification of the pyrethroids bifenthrin, α-cypermethrin, β-cyfluthrin, and τ-fluvalinate (Niu et al. 2012, Demkovich et al. 2015a) as well as in bifenthrin resistance in navel orangeworm populations in Kern County, California (Demkovich et al. 2015b). The insecticides sprayed to control navel

orangeworm in heavily infested orchards are usually applied in rotation during the growing season (Niu et al. 2012); rotational patterns may actually increase the risk of cross resistance if different families of insecticide share a common route of detoxification. Kirst (2010) noted that there have been multiple cases of resistance to spinosyns, but the underlying mechanism of resistance varies with the taxon. Wang et al. (2016) reported that resistance to spinosad in *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) is associated with a mutation of the α6 subunit of the nicotinic acetylcholine receptors, confirming findings in *Drosophila* knockout studies in previous years (Perry et al. 2007). Hamby et al. (2015) found that another spinosyn, spinetoram, was effective at reducing populations not only of navel orangeworm but also the peach twig borer *Anarsia lineatella* (Zeller) (Lepidoptera: Gelechiidae) after a single spring application.

Like spinosyns, neonicotinoid insecticides act as an agonist on the nicotinic acetylcholine receptor (Tomizawa and Casida 2005). They were introduced to the market in the 1990s and have been particularly effective against piercing and sucking pests such as planthoppers and psyllids. The neonicotinoid acetamiprid is not directly applied against *A. transitella*; rather, it is applied against other pests, including Gill's mealybug *Ferrisia gilli* (Gullan) (Hemiptera: Pseudococcidae) (Haviland et al. 2012) and peach twig borer (Zalom et al. 2012), at times when the navel orangeworm is likely to be present. Clothianidin, a neonicotinoid with similar chemistry to acetamiprid, is used on almonds and other tree nuts as Belay® Insecticide from Valent. Nauen et al. (2003) found that in both cotton plants and their pest *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) clothianidin is a degradation product of the neonicotinoid thiamethoxam, which has been used on almond crops as recently as 2013 (CDPR 2014). The neonicotinoid imidacloprid although not used on almonds since 2008, is still used on many crops

in the Central Valley of California including pistachios, a crop susceptible to infestation by navel orangeworm (CDPR 2014).

Determining what detoxification mechanisms are utilized by navel orangeworm against these insecticides is an important step in avoiding the development of insecticide resistance. In this study, I investigated the toxicity and detoxification of the neonicotinoid insecticides acetamiprid, imidacloprid, and clothianidin, as well as the organic insecticide spinosad, by examining the effects of the synergists piperonyl butoxide and diethyl maleate on insecticide toxicity. These synergists are used as tools to differentiate among classes of detoxification enzymes; piperonyl butoxide inhibits primarily P450s (Hodgson and Levi 1998, Demkovich et al. 2015a) and diethyl maleate inhibits primarily glutathione-S-transferases (Welling and de Vries 1985).

Materials and Methods

Insecticide/diet preparation

The insecticides acetamiprid, clothianidin, imidacloprid, and spinosad as well as the synergist diethyl maleate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synergist piperonyl butoxide (PBO) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Insecticides were suspended in acetone prior to addition to unsolidified artificial diet. The artificial diet used is formulated for lepidopterans (based on Waldbauer 1984) and once mixed is poured into individual 28 mL (one-ounce) plastic cups (Solo Cup Company, Dart Container, Mason, MI, USA).

Navel orangeworm colony

Colonies of navel orangeworm were maintained on a wheat bran diet as described in Tebbets et al. (1978) and Finney and Brinkman (1967). Eggs of *A. transitella* were obtained from

the Commodity Protection and Quality Unit of USDA (Parlier, CA, USA) from a stock that originated from almond trees in Fresno, CA, USA but has been maintained for over 25 years in laboratory culture (designated the CPQ strain). After egg hatch, first instar caterpillars were placed on a semi-synthetic artificial diet described above.

LC_{50} bioassays

Bioassays were conducted with first instar navel orangeworm (NOW) caterpillars of the CPQ strain. Eggs were collected daily from adult mating chambers and kept in moist bags until hatch. First instar caterpillars more than 48 hours old were not used in assays. Caterpillars were moved gently by paintbrush onto the artificial diet containing the treatment – four individuals in each diet cup. There were five replicate cups for each treatment, and the experiment was repeated at least three times. Number of individuals dead at 48 hours were counted by gently prodding caterpillars with a paintbrush and waiting for movement. Five concentrations were needed for successful Probit analysis and adjustments to concentration ranges tested were made after initial trials to more closely bound the potential LC_{50} value. Concentrations used and mortality data used for Probit analysis can be seen in Table 1.2.

Synergism experiments

Concentrations that fell within the 95% confidence interval of the calculated median-lethal concentrations for each pesticide (25 µg/g acetamiprid; 100 µg/g clothianidin; 1 µg/g spinosad) were used for synergism assays with PBO (200 µg/g) and DEM (200 µg/g). Control treatments were run with both PBO and DEM to determine whether control toxicity was close to or equal to zero mortality at the highest concentration possible. Assays were similar to those used to assess LC_{50} values as described earlier. In the case of acetamiprid, PBO and DEM trials were conducted at separate times as a result of limited availability of first instars and thus were

analyzed separately. Maximum concentrations of PBO and DEM that resulted in no mortality were determined previously (Niu 2010).

Statistical analyses

Probit analysis (SPSS version 22, SPSS Inc., Chicago, IL, USA) was used to determine the median-lethal concentrations after 48 hours for all insecticides. For each Probit model, the Pearson Goodness-of-Fit Test was used to confirm that the dataset met the assumptions of the model, and if assumptions were not met a heterogeneity factor was used in the calculation of the confidence limits. Regression analysis was used to assess differences in the rate of mortality between treatments and to insure that control treatments using DEM or PBO did not cause significant mortality when compared to controls, and differences in the slopes were assessed according to Zar (1984).

Results

Spinosad was more toxic to first instar *A. transitella* than any neonicotinoid tested, with an LC_{50} of 2.32 µg/g (95% CI 1.09-6.85 µg/g) (Table 1.1). The most toxic neonicotinoid acetamiprid was an order of magnitude less toxic than spinosad, with an LC_{50} value of 26.56 µg/g (95% CI 21.04 – 32.40 µg/g). Clothianidin had an LC_{50} of 107.5 µg/g (95% CI 83.19-141.36 µg/g) – one-quarter the toxicity of acetamiprid. The neonicotinoid imidacloprid was even less toxic to the extent that it could not be used for inhibitor assays or to determine an LC_{50} – on diet with 100 µg/g imidacloprid, mortality was only 10% after 48 hours. These insecticide mortality data fit the Probit model as indicated by the goodness-of-fit test ($P > 0.05$) for acetamiprid. In the case of both clothianidin and spinosad, a heterogeneity factor was applied in SPSS to calculate the confidence intervals.

A comparison of slopes showed that in the case of spinosad PBO synergized (t = 2.578; df = 52; P = 0.016) the toxicity of the insecticide, increasing the toxicity by an average of 57.6%; the difference in toxicity with the addition of DEM was not significant (t = 1.659; df = 52; P > 0.05) (Table 1.3, Figure 1.1). Acetamiprid toxicity was increased in the presence of PBO (t = 4.611; df = 52; P < 0.001) and 79.6%, but not with DEM (t = 0.521; df = 52; P > 0.05) (Table 1.3, Figure 1.2). Clothianidin toxicity was not significantly changed in the presence of either DEM (t = 0.578; df = 52; P > 0.05) or PBO (t = 0.002; df = 52; P > 0.05) (Table 1.3, Figure 1.3).

Discussion

There are currently multiple insecticides registered for control of lepidopteran and hemipteran pests in California almond orchards (Niu et al. 2012). As mentioned, although the neonicotinoid acetamiprid is not directly applied for use against navel orangeworm, it is applied to manage other almond pests, including Gill's mealybug (Haviland et al. 2012) and peach twig borer (Zalom et al. 2012) at times when navel orangeworm is present. Peach twig borer, an important pest of almonds, feeds on new crop nuts, and insecticides used for its control (including acetamiprid) can result in navel orangeworm exposure to additional insecticides. In almond orchards where both insects are a concern, the peach twig borer spray is also timed to cover navel orangeworm spring activity (Zalom et al. 2012, Hamby and Zalom 2013). Here I have demonstrated that acetamiprid, a neonicotinoid, has some navel orangeworm activity as well, even though navel orangeworm is not listed on its label. I have also shown that PBO synergizes the toxicity of acetamiprid, consistent with the findings of Ninsin and Tanaka (2005) demonstrating synergism with PBO in a resistant laboratory colony of diamondback moth and indicative of P450-mediated detoxification of this neonicotinoid in navel orangeworm. In the case of the much less toxic clothianidin, no increases in mortality were seen with the addition of

either PBO or DEM, indicating that P450s and glutathione-S-transferases do not play a large role in the metabolism of this particular neonicotinoid. It should be noted that both clothianidin and imidacloprid are substantially less toxic to navel orangeworm than acetamiprid.

Spinosyns are the only insecticides examined in this study registered for use in organic orchards, where they are marketed under the trade name Entrust® or as Spinosad. In conventional agriculture, spinosyns are applied as the sole ingredient (Delegate®) or as a premixed with another insecticide (spinetoram-methoxyfenozide—Intrepid-Edge®). Even with a novel mode of action, multiple cases of resistance to spinosyns, involving different resistance mechanisms, have been reported in Lepidoptera (Moulton et al. 2000, Zhao et al. 2002, Sayyed et al. 2008). In a resistant strain of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), PBO synergized spinosad, which suggests the involvement of P450s (Wang et al. 2006). I found similar evidence of synergism with PBO in the case of spinosad in navel orangeworm, indicating that P450s are involved in detoxification of this particular spinosyn. Glutathione-S-transferases are likely not involved in the detoxification of spinosad as I did not find that diethyl maleate significantly influenced the toxicity of spinosad.

The rapid expansion of almond and pistachio acreage (hectarage) over the past decade places these new orchards adjacent to citrus, cotton, grapes, and stone fruit, which in turn increases the probability of nontarget exposure of navel orangeworm to insecticides as a result of drift. Some of these insecticides are not intended for control of navel orangeworm but exposure to their active ingredients may increase selection pressure on the navel orangeworm for resistance to multiple classes of insecticide (Higbee and Siegel 2009). Given my findings that P450s play a role in the detoxification of both spinosad and acetamiprid, inhibiting the P450 enzymes involved in the detoxification of insecticides (Lee and Campbell 2000, Niu et al. 2012)

may be a valid strategy for navel orangeworm management, particularly in populations where P450-mediated resistance is a problem (e.g., Kern County, California, where bifenthrin resistance has appeared, Demkovich et al. 2015b). However, piperonyl butoxide, a P450 inhibitor, is currently not registered for use in tree crops, such as almonds; there may be a future need for an approved synergist for insecticides used in tree nut crops if resistance becomes widespread.

Tables and Figures

Table 1.1. Insecticide classifications, commercial names, and modes of action for acetamiprid, clothianidin, imidacloprid, spinosad, piperonyl butoxide, and diethyl maleate. Initial bioassay LC_{50} values along with 95% confidence interval were determined by Probit analysis data for acetamiprid, clothianidin, and spinosad in a laboratory strain of navel orangeworm (CPQ).

Active Ingredient	Commercial Name(s)	Chemical Family	Mode of Action	IRAC Group/ Subgroup	LC_{50} (µg/g) and 95% CI
Acetamiprid	Assail®	Neonicotinoid	Nicotinic acetylcholine receptor modulator	4A	26.56 (21.04 - 32.40)
Clothianidin	Belay® Poncho®	Neonicotinoid	Nicotinic acetylcholine receptor modulator	4A	107.5 (83.19 - 141.36)
Imidacloprid[a]	Admire Pro®	Neonicotinoid	Nicotinic acetylcholine receptor modulator	4A	N/A
Spinosad	Conserve® Entrust®	Spinosyn	Nicotinic acetylcholine receptor modulator; GABA neurotransmitter agonist	5	2.32 (1.09 - 6.85)
Piperonyl butoxide	Butacide® Incite® Butoxide®	Synergist	Cytochrome P450 monooxygenase inhibitor	N/A	N/A
Diethyl maleate		Synergist	Glutathione-S-transferase inhibitor	N/A	N/A

[a] Although not currently approved for use in almonds, this product is used in pistachio orchards where the navel orangeworm is also present.

Table 1.2. Information below was used to calculate the LC_{50} concentrations used in synergism assays. Number of dead navel orangeworm first instars (r) out of total (n) were counted after 48 hours of treatment.

Acetamiprid

	0 µg/g	5 µg/g	25 µg/g	50 µg/g	100 µg/g	200 µg/g
r	5	2	15	48	54	59
n	137	60	35	59	60	60

Clothianidin

	0 µg/g	1 µg/g	5 µg/g	10 µg/g	25 µg/g	50 µg/g	100 µg/g	200 µg/g	500 µg/g	750 µg/g
r	1	0	1	5	15	28	30	41	44	31
n	60	20	58	39	58	75	60	60	59	40

Spinosad

	0 µg/g	0.1 µg/g	0.5 µg/g	1 µg/g	2 µg/g	2.5 µg/g	4 µg/g	8 µg/g
r	5	9	14	16	22	83	57	61
n	114	77	79	79	79	139	80	80

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Table 1.3. Regression data for synergist bioassays monitoring mortality over time with the insecticides acetamiprid, spinosad, and clothianidin as well as synergists diethyl maleate (DEM) and piperonyl butoxide (PBO) in a laboratory strain of navel orangeworm (CPQ). Amounts of DEM and PBO were standard across treatments; both were kept constant at 200 µg/g. Log time in the equation represents when the $\log_{10}$ was taken in order to have a better fit regression equation (higher R^2 value).

Treatment	Equation	R^2	F	*P*
Acetamiprid PBO Control	Mortality = -0.0954 + 0.0863 * log time	0.40	(1, 27) = 17.63	0.0003
25 µg/g Acetamiprid	Mortality = -0.5555 + 0.5125 * log time	0.73	(1, 27) = 70.31	<0.0001
25 µg/g Acetamiprid + PBO	Mortality = -0.9312 + 0.9204 * log time	0.89	(1, 27) = 206.97	<0.0001
Acetamiprid DEM Control[a]	Mortality = -0.0569 + 0.0487 * log time	0.23	(1, 20) = 5.68	0.0277
25 µg/g Acetamiprid[a]	Mortality = -0.2762 + 0.3057 * log time	0.53	(1, 20) = 21.64	0.0002
25 µg/g Acetamiprid + DEM[a]	Mortality = -0.4048 + 0.4737 * log time	0.54	(1, 20) = 22.59	<0.0001
Clothianidin PBO Control	Mortality = -0.0158 + 0.0006 * time	0.31	(1, 27) = 11.50	0.0022
Clothianidin DEM Control	Mortality = -0.0047 + 0.0001 * time	0.14	(1, 27) = 4.32	0.0477
100 µg/g Clothianidin	Mortality = -1.0920 + 0.9026 * log time	0.92	(1, 27) = 288.97	<0.0001
100 µg/g Clothianidin + PBO	Mortality = -0.0828 + 0.0008 * time	0.86	(1, 27) = 154.25	<0.0001
100 µg/g Clothianidin + DEM	Mortality = -0.8759 + 0.7178 * log time	0.84	(1, 27) = 137.15	<0.0001
Spinosad PBO Control[b]	Mortality = 0 + 0 * time	N/A	N/A	N/A
Spinosad DEM Control[b]	Mortality = -0.0286 + 0.0015 * time	N/A	N/A	N/A
1 µg/g Spinosad	Mortality = -0.0585 + 0.0043 * time	0.80	(1, 27) = 101.46	<0.0001
1 µg/g Spinosad + PBO	Mortality = -0.977 + 0.8058 * log time	0.74	(1, 27) = 75.66	<0.0001
1 µg/g Spinosad + DEM	Mortality = -0.0615 + 0.0052 * time	0.90	(1, 27) = 236.68	<0.0001

[a] Acetamiprid synergist trials were run separately and thus are analyzed separately.
[b] There was no significant regression relationship ($P > 0.05$).

Figure 1.1. Effect of adding inhibitors piperonyl butoxide (PBO) and diethyl maleate (DEM) to artificial diet containing spinosad on mortality of first instar navel orangeworm caterpillars. Error bars represent the standard error.

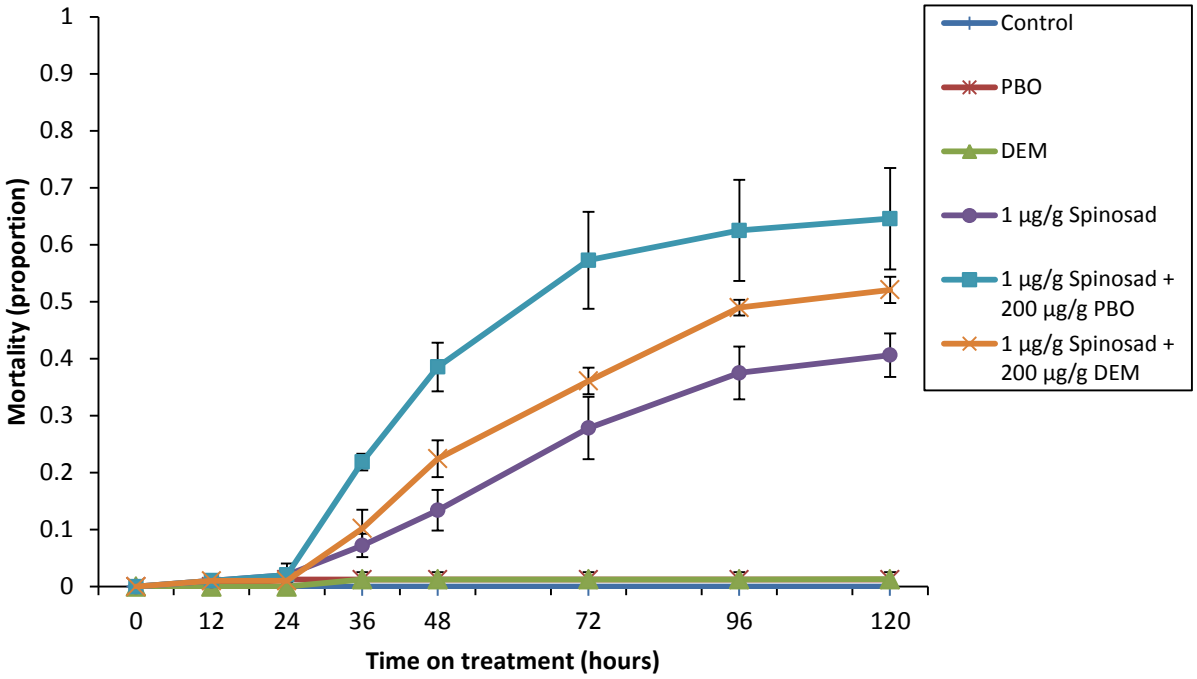
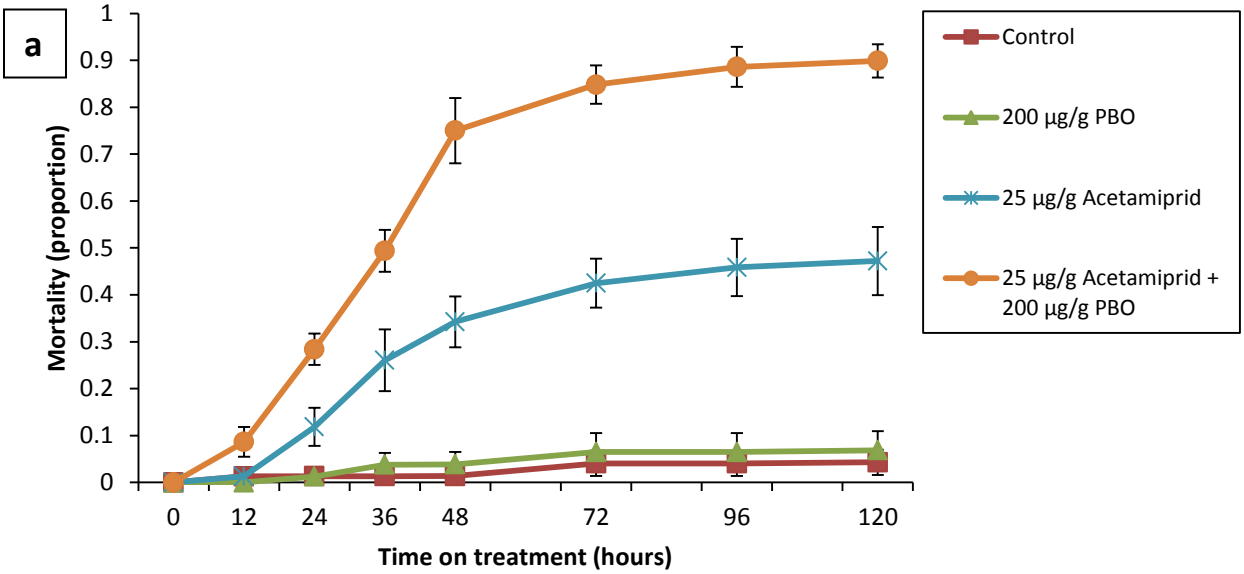


Figure 1.2. a. Effect of piperonyl butoxide (PBO) on mortality of newly hatched navel orangeworm caterpillars exposed to acetamiprid-containing artificial diets. These diets contained 25 µg/g acetamiprid supplemented with or without 200 µg/g PBO. **b.** Effect of diethyl maleate (DEM) on mortality of newly hatched navel orangeworm caterpillars exposed to artificial diet with acetamiprid. Standard error bars are included for each treatment.



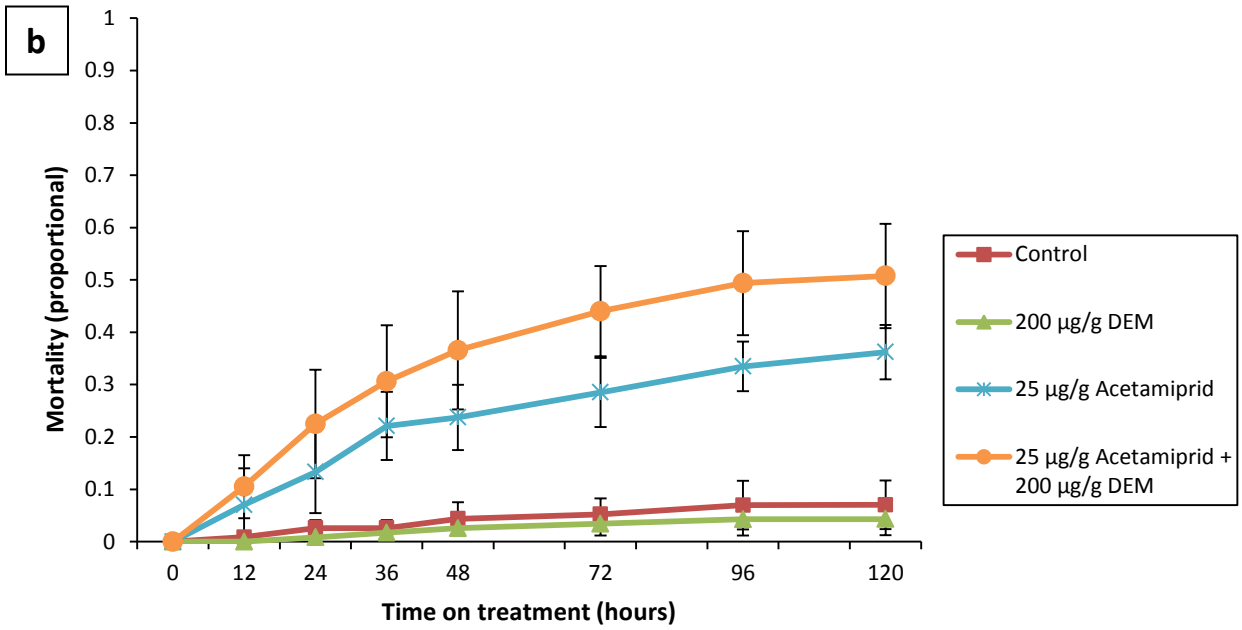
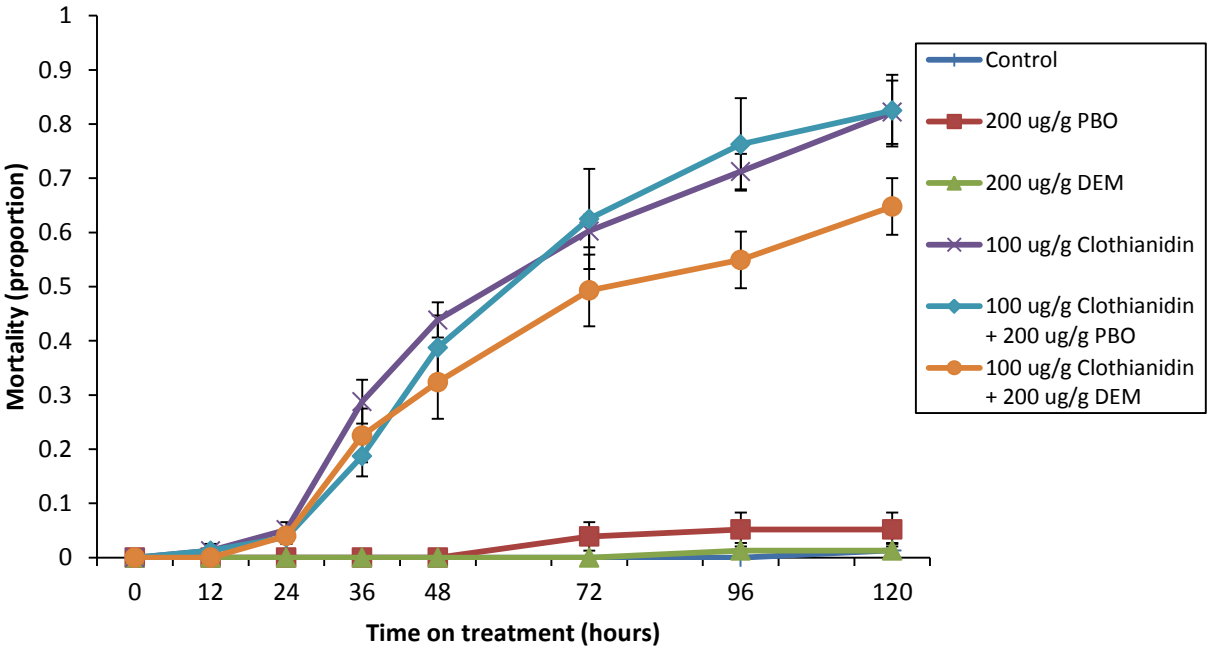


Figure 1.3. Effect of adding inhibitors piperonyl butoxide (PBO) and diethyl maleate (DEM) to artificial diet containing clothianidin on mortality of first instar navel orangeworm caterpillars. Standard error bars are included for each treatment.



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II. Behavioral responses of free-flying honey bees to the presence of dietary neonicotinoids

Introduction

Neonicotinoids were introduced as insect control agents in the mid-1990s and are popular because of their low toxicity to birds and mammals compared to many other available insecticides. By binding irreversibly to nicotinic acetylcholine receptors in the central nervous system, this class of insecticides causes overstimulation, paralysis, and death in insects (Tomizawa and Casida 2005). Neonicotinoids are typically delivered as systemic pesticides in agroecosystems, which means they are absorbed and incorporated into plant tissues, subsequently providing protection from insects that feed on vascular fluids as well as those that chew foliage. However, the quantity of neonicotinoids found in plant tissue varies by location within the plant; imidacloprid and its respective metabolite residues in pollen were higher in concentration than those found in nectar by an average of 80% (Dively and Kamel 2012).

Complicating the process of assessing environmental risks to nontarget beneficial insects, including pollinators is the variety of neonicotinoids on the commercial and domestic market with differential toxicity to beneficial species. Assessing these risk factors in honey bees (*Apis mellifera*) is particularly difficult -- sublethal effects of neonicotinoid pesticides on honey bees have been the focus of several well-publicized studies on honey bee decline. Neonicotinoid impacts on honey bees include reduced rates of foraging, slower return of foragers to the hive, decreased recruitment, higher rates of queen replacement, interference with olfactory associative and visual learning, and impaired motor function (Henry et al. 2012, Eiri and Nieh 2012, Dively et al. 2015, Williamson and Wright 2013, Williamson et al. 2014). Neonicotinoids can also interfere with the ability of worker bees to deal with other stressors in their environment, by lowering their immune response (Brandt et al. 2016), and influencing their susceptibility to

pathogens (Alaux et al. 2010) and *Varroa* infestations (Dively et al. 2015). Colony-scale impacts have been harder to assess, and at least one study has shown little to no causal relationship between colony mortality and imidacloprid exposure at field-relevant concentrations (Chauzat et al. 2009).

Imidacloprid is no longer approved for use in California almond orchards, following the voluntary request of the Environmental Protection Agency from the manufacturer Bayer to remove almond application from product labels in 2010. The less toxic neonicotinoid acetamiprid continues to be used in almond orchards (CDPR 2014) to control mealybugs and peach twig borer, *Anarsia lineatella* (Zeller) (Lepidoptera: Gelechiidae) (Zalom et. al 2012). While most insecticides are not applied during bloom, honey bees might become exposed to acetamiprid from products applied earlier in the growing season; neonicotinoids and their metabolites can remain in woody plants and soils for over a year in some cases (Bonmatin et al. 2015, Cowles et al. 2006).

Although many studies have addressed the sublethal effects of pesticides in honey bees, there have been far fewer that examine how and when honey bees naturally encounter these pesticides in their environment and whether, under natural conditions, they can detect and avoid food contaminated with neonicotinoids. Although honey bees are broadly polylectic, they possess few gustatory receptor genes relative to other insects (Robertson and Wanner 2006); nevertheless, free-flying foragers are capable of detecting a diversity of toxins in nectar sources and displaying learned avoidance responses to potential toxins (Wright et al. 2010). Honey bees display a dose-dependent response to many nectar secondary metabolites, including nicotine and caffeine (Detzel and Wink 1993, Singaravelan et al. 2005, Wright et al. 2013). Moreover, they display paradoxical preferences for certain neurotoxic compounds at low concentrations that, at

high concentrations, are deterrent (Singaravelan et al. 2005, Hagler and Buchmann 1993). Assessing whether honey bees can detect and avoid pesticide-contaminated nectar or pollen is further complicated because most behaviorial bioassays are conducted under highly artificial conditions, including no-choice assays or immobilization (e.g., Ayestaran et al. 2010); bees thus are not able to engage their full behavioral repertoire (Berenbaum 2015). The Environmental Protection Agency convened a Scientific Advisory Panel in 2012 to identify "data gaps" related to assessing risks of exposure to neonicotinoid and other systemic pesticides. The incomplete knowledge of natural feeding behaviors in the presence of ecologically realistic field concentrations of pesticides was considered such a gap.

Nicotine, a naturally occurring neurotoxin in nectar of *Nicotiana tabacum* and several other plants, has been shown in experiments with free-flying honey bees to elicit a feeding preference (Singaravelan et al. 2005) or only partial repellency (Köhler et al. 2011) at naturally occurring concentrations. Partially repellent nectars have been hypothesized to enhance cross-pollination by encouraging pollinators to move between flowers more rapidly and potentially reduce self-fertilization that can occur when the pollinator remains at a single flower or plant for an extended period of time (Adler 2000). This avoidance could be an important consideration given the increased use of synthetic neonicotinoid pesticides in agricultural and domestic systems. Neonicotinoids and nicotine both bind to nicotinic acetylcholine receptors in the central nervous system. In the case of neonicotinoids, binding is irreversible, causing overstimulation, paralysis, and death in insects. Although there are numerous studies demonstrating behavioral effects of neonicotinoids under assay conditions involving immobilization and/or force-feeding (e.g., Eiri and Nieh 2012, Laycock and Cresswell 2013, Laycock et al. 2014, Williamson et al. 2014) there is a need for more realistic, ecologically appropriate field studies (Henry et al. 2012,

Cresswell and Thompson 2012). To date, only a single study (Kessler et al. 2015) has examined feeding preferences in free-flying bees given free-choice nectar-relevant concentrations of neonicotinoid pesticides. Kessler et al. (2015) found that honey bees displayed a preference for solutions with imidacloprid and thiamethoxam over unadulterated sucrose solutions, despite the fact that there is no electrophysiological evidence that bees can detect these compounds. One problematic aspect of this study is that honey bees, while capable of flying short distances, were enclosed in 11 x 6 x 20 cm plastic boxes during assays, which necessarily constrained their range of behaviors.

In the absence of an established method for assessing behavior of free flying bees, I designed and tested a system, then conducted a series of feeding preference bioassays using free-flying foragers under conditions more closely approximating field conditions. I examined two neonicotinoids, imidacloprid and acetamiprid, at a range of concentrations in sugar water that was not significantly toxic to honey bees, including field-relevant concentrations.

Materials and Methods

Research colonies

Research hives of *Apis mellifera* were assembled immediately before placement in an outdoor shade structure. For each hive, five frames from existing healthy hives were placed in a single box from the Bee Research Facility, University of Illinois Urbana-Champaign, IL, USA. Queens were kept caged inside the hive for 7 to 10 days before release.

Insecticide preparation

Quinine, acetamiprid, and imidacloprid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Insecticides were dissolved in methanol before being added to 30% sugar water

(weight/volume) solutions. Both insecticide and solvent control sugar water solutions contained 2 µl/mL methanol.

Equipment

The scale used at each feeding station was a 2000G/0.01G B20002T Electronic Balance Laboratory Scale (Olymstore, Amazon.com). The cameras used were Vimtag (Fujikam) 361 HD (Vimtag, Amazon.com). Feeding chambers (Figure 2.1) were custom-made using 6.4 mm (1/4 inch) thick acrylic sheets to form a box with a removable lid; eight holes were cut into one side of the box and attached to these openings were 9.5 mm (3/8 inch) diameter acrylic tubes glued in place using SciGrip Plastic Pipe Cement (McMaster-Carr, Elmhurst, IL, USA). These tubes allowed for videotaping the movements of bees in and out of the box.

Flight chamber conditions

Free-flight trials were carried out in an outdoor shade structure 18.3 meters by 6.1 meters (60 feet by 20 feet) divided lengthwise into two arenas (18.3 meters by 3.0 meters) by shadecloth. Experiments were conducted during the months of July through September 2015. The shade structure is located in Urbana, Illinois, adjacent to the University of Illinois Pollinatarium (latitude/longitude: 40.087108 ºN, 88.214582 ºW). Both freshly ground pollen granules (Bee Pollen Whole Granules, Y.S. Organic Bee Farms, Sheridan, IL, USA) and water were supplied and replaced daily in dishes directly adjacent to the entrance of the hive. Before trials were conducted, honey bees were seen visiting both resources regularly. Hives were placed in the center of the shade structure arena with feeding stations at either end of the arena (both 7 meters away from hive entrance). Trials were run during daylight hours, when there was no rain, and when the temperature was higher than 20ºC. If rain began during an experiment, cameras and treatments were removed.

The feeder consisted of a 148 mL (5-ounce) cup filled with sugar water inverted on a dish with etched feeding guides that allowed for feeding with minimal evaporation. After completion of training to feeders, the feeder was enclosed in clear acrylic box with entrance tubes such that bees could be counted when entering and exiting (Figure 2.1). This design also prevented other insects (ants and flies) from taking advantage of the sugar water source and thus displacing foraging honey bees.

Sugar water feeder training (initial)

Once hives were placed in the shade structure, the bees were trained to feeders over the next two to three days. Bees were trained to two unadulterated 30% sugar water (w/v) feeders by placing the feeders in front of the hive entrance for several hours. If feeding was not seen after several hours, honey was used to attract the bees to the feeder. Once bees started feeding, the base and lid of the feeder housing were added over the course of several hours. Once bees were acclimated to each feeder, feeders were moved in tandem toward either end of the arena over the course of several hours.

Free-flight experiment

Pesticide and quinine concentrations used in free-flight assays were chosen based on caged trials in which no statistically significant mortality was seen over 48 hours when bees were fed pesticide-laden sugar water *ad libitum*. This study focused on two neonicotinoids: imidacloprid, which is widely used as a systemic and is highly toxic to honey bees, and acetamiprid, with markedly lower toxicity (Blacquière et al. 2012). For imidacloprid, 5 ng/mL was used based on the high toxicity seen in initial small cage trials (unpublished data) and previous literature (Table 2.1). Concentrations between 5 ng/mL and 50 ng/mL fall within likely field-relevant concentrations of neonicotinoids (Dively and Kamel 2012, Blacquière et al. 2012).

Concentrations chosen for free flight assays for acetamiprid were 50 ng/mL, 500 ng/mL and 5 µg/mL. Quinine was used in a preliminary experiment to establish this method as an effective way to assay behavioral responses (Table 2.1) and determine a time interval sufficient for determining a behavioral response. Quinine is known to be aversive to honey bees and is frequently used in learning and memory studies in honey bees (Wright et al. 2010, Perry and Barron 2013, Avarguès-Weber et al. 2010).

Once bees were trained to feeder stations, trials consisted of 30% sugar water placed at both feeding stations. Trials were videotaped for 30 – 60 minutes to obtain baseline foraging data and allow scout bees to find feeders. Next, new feeders were placed into position to replace the training feeders. These contained either the treatment (pesticide in 30% sugar water) or the control (30% sugar water with methanol). Once these feeders had been recorded and were in place for 60 minutes, their positions were switched and the feeders were again videotaped for 60 minutes. Finally, feeders were switched out with unamended sugar water to obtain another set of baseline foraging data. Feeder weight was continuously monitored by having each station on a scale so the weight of sugar water consumed could be recorded. Also, the number of bees entering and exiting each station was recorded by video camera. Each concentration of pesticide was tested four times (six in the case of imidacloprid), and pairs of replicates were run in tandem to avoid for positional bias from the sun's position (treatment placed on west side for one hive and east side for the second hive),

Data collection and statistical analyses

Individual honey bees were counted using image stills from videos with the multipoint tool in ImageJ Version 1.50i (NIH, USA) (Figure 2.2). In cases where image stills were unclear,

the video was examined and if necessary the numbers of bees entering and exciting the arena were counted between time points.

The quinine preliminary trial data were used to determine the length of time after switching feeders when significant differences could be detected in the number of bees visiting the feeder with the use of a paired t-test using JMP Version 12.2.0 (SAS Institute Inc., Indianapolis, IN, USA). Once determined, this unit of time was used for the analysis of foraging rates with the neonicotinoid insecticides.

Results

Preliminary trials with quinine revealed repellency using our methodology in free-flight experiments (Figure 2.3). Paired t-tests were used to compare the number of bees at each feeder for every minute after placement; after 9 minutes there were two to three times more honey bees on the sugar water control feeders than on the quinine feeders ($P = 0.0001$) (Table 2.2). Through this negative control trial, we determined 10 minutes to be an interval sufficient to detect significant differences in bee visitation at the feeders in subsequent experiments, although each trial was run for 60 minutes.

In the experiment comparing feeders with 5 ng/mL imidacloprid to feeders with unamended sugar water, 1.3 times more bees were visiting the feeder containing the treatment at 9 minutes ($P = 0.0442$, paired t-test) and 10 minutes ($P = 0.0197$, paired t-test) after placement (Table 2.3, Figure 2.4). This trend can be seen in Figure 2.4, where in five of the six trials differences between treatment and control were evident after approximately 8 minutes. Measurements of weight data proved unsuccessful due to difficulties with equipment and variability in evaporation rates.

The presence of acetamiprid in feeders at any of the concentrations tested (50 ng/mL, 500 ng/mL, and 5 µg/mL) did not produce differences in foraging between the control and treatment feeders after 10 minutes of foraging ($P > 0.05$, paired t-test) (Table 2.4) nor were any trends evident over the full duration of the experiment overall in each of the four trials run for each concentration (Figure 2.5, Figure 2.6, Figure 2.7).

Discussion

Quinine, a naturally occurring alkaloid, found in *Cinchona* spp. (Rubiaceae) (Staba and Chung 1981), is repellent to honey bees under the conditions of our experimental design. Given the choice between sugar water and sugar water containing 15 mM quinine, free-flying honey bees chose the feeder without the bitter alkaloid by a factor of two- to three-fold after only nine minutes. Similar experiments have been carried out with other secondary plant metabolites that may be found in nectar at low concentrations. Singaravelan et al. (2005) found that free-flying bees were attracted to a variety of phytochemicals at low (and realistic) concentrations and deterred by higher concentrations. Among these phytochemicals were nicotine and caffeine, both neuroactive and both toxic to honey bees at high concentrations (Detzel and Wink 1993). Wright et al. (2013) found that, when caffeine is present at low concentrations in a sucrose solution, it can enrich the memory of the reward. If insecticides, such as the neonicotinoids, act similarly in nectar as do neuroactive phytochemicals, understanding the concentration ranges at which memory enhancement occurs is important in understanding risks associated with using these insecticides on honey bee-pollinated crops. In fact, there are studies demonstrating that imidacloprid can facilitate learning (Lambin et al. 2001) and enhance memory when applied in combination with the pesticide coumaphos (Williamson et al. 2013).

I found that honey bee foragers showed a preference for sugar water containing imidacloprid at 5 ng/mL over the control (Table 2.3, Figure 2.4). Kessler et al. (2015) found that in small cage studies honey bees were also attracted to imidacloprid over the control, although in their study they tested the effects of this pesticide at higher concentrations, 25.6 ng/mL and 256 ng/mL. They did report feeding stimulation by another neonicotinoid, thiamethoxam, at concentrations similar to those used in my study, with lower limits reaching 2.6 ng/mL.

Imidacloprid is so highly toxic to honey bees that even at the seemingly low and field realistic 5 ng/mL concentration used in this study a preference for feeding on contaminated nectar could result in ingestion of this pesticide at levels causing sub-lethal toxicity. If honey bees carry a load of between 25 and 75 µl of nectar (or sugar water) in their honey stomach (Seeley 1985, Gary 1992, Lundie 1925), this would expose them to between 125 to 375 picograms per trip, which over the course of 10 trips would cumulatively expose them to amounts at the lower range of the oral 48-hour LD_{50} of imidacloprid, 3.7 ng/bee (Blacquière et al. 2012). While foragers are likely regurgitating much of this volume when they return to the hive, studies of neonicotinoids at field-relevant concentrations or higher might reflect consequences of exposures at the level of the oral LD_{50}. Imidacloprid at sublethal concentrations can cause slow return of foragers to the hive (Henry et al. 2012), and decrease recruitment (Eiri and Nieh 2012). Notwithstanding, my results show that imidacloprid is attractive even at concentrations that may cause sublethal toxicity.

As Kessler et al. (2015) reported for clothianidin, I found no preference for or aversion to acetamiprid over a range of concentrations (Table 2.4; Figures 2.5, Figure 2.6, Figure 2.7). Both acetamiprid and thiacloprid are neonicotinoids with relatively low toxicity to honey bees, with LD_{50} values for these two pesticides greater than 100 times those of other commercially used

neonicotinoids, such as imidacloprid, clothianidin, and thiamethoxam (Iwasa et al. 2004). I attempted to control environmental and situational factors as much as possible in this study by conducting trials when weather allowed, by avoiding the use of colors or other symbols as markers to avoid preference or learning cues, and by replicating experiments in tandem to avoid any impacts from the position of the sun on treatment location. It is possible that weather or other factors masked preference behavior, but this is also a reminder that the foraging choices that honey bees make are complex and influenced by a multitude of factors.

Because acetamiprid has such low toxicity, I was able to test its effects on behavior at a wide range of concentrations (50 ng/mL, 500 ng/mL, and 5 µg/mL) (Table 2.1). Concentrations of particular neonicotinoids found in nectar in the field are not well characterized despite their importance in honey bee health risk assessments. Dively and Kamel (2012) found imidacloprid residues as high as 122 ng/g in pollen and 17.6 ng/g in nectar in pumpkin (*Cucurbita pepo* L. var. 'Howden'), a crop where soil drenching or spray-and-drip irrigation pesticide application is frequently used and results in higher concentrations of neonicotinoids in nectar and pollen (Stoner and Eitzer 2012). Amounts of neonicotinoids in seed-treated crops, such as sunflower, canola, and corn, may not accurately reflect systemic amounts of pesticides in other crops with different methods of pesticide application and thus make risk assessment difficult (Lundin et al. 2015). Residue studies on woody plants focus on concentrations found in xylem and phloem and studies that do examine concentrations in nectar are primarily on annual and not perennial plants (Blacquière et al. 2012, Bonmatin et al. 2015).

Much attention has been focused on nontarget impacts of neonicotinoids on pollinators in almond orchards; the lack of knowledge about insecticide concentrations in nectar of almond trees treated with acetamiprid makes identifying field-realistic concentrations and assessing risks

of honey bee exposure difficult if not impossible. This information is vital for understanding the implications of the results presented here and elsewhere (Kessler et al. 2015) on the attractive nature of particular insecticides. Future studies should measure concentrations of insecticides found not only in almonds but in a variety of crops, including nut crop or fruit orchards, where floral choices are often limited for honey bees.

Tables and Figures

Table 2.1. An introduction to the compounds used in this study and their LD_{50} information and concentrations in sugar water that were used in this study.

Compound	**Insecticide Class**	**Mode(s) of application**	**Commercial name(s)**	**48h LD_{50} (oral)**	**Concentration(s) used in this study**
Imidacloprid	Neonicotinoid	Not currently used	Admire Pro®	3.7 – 60 ng/bee[a,b,c]	5 ng/mL 50 ng/mL
Acetamiprid	Neonicotinoid	Soluble granule for soil treatment; Wettable powder for spray	Assail®	14.53 µg/bee[a]	50 ng/mL 500 ng/mL 5 µg/mL
Quinine	Natural product	N/A	N/A	N/A	15 mM[d]

[a] Blacquière et al. 2012
[b] Suchail et al. 2001
[c] Decourtye and Devillers 2010
[d] Concentrations used based on Wright et al. 2010

Table 2.2. Raw counts of bees visiting feeders after switching from sugar water to treatment (quinine). Time represents how long after switching feeders from initial sugar water to treatment, 15 mM quinine (Q), or sugar water control (S). A significantly greater average count is bolded. Significance values from paired t-test greater than p>0.05 are indicated by NS.

	1 min		2 min		3 min		4 min		5 min		6 min		7 min		8 min		9 min		10 min		25 min	
	Q	S	Q	S	Q	S	Q	S	Q	S	Q	S	Q	S	Q	S	Q	S	Q	S	Q	S
Trial 1	6	6	6	5	5	9	7	10	5	11	5	6	13	6	11	16	4	15	5	12	8	19
Trial 2	18	12	11	16	8	10	9	9	14	9	10	19	9	23	12	27	13	26	9	23	6	23
Trial 3	3	2	7	6	7	11	8	15	8	23	19	22	10	17	9	19	15	26	16	32	13	36
Trial 4	5	2	6	5	3	11	5	5	3	9	4	7	3	12	2	5	2	14	1	13	3	14
Average	8.0	5.5	7.5	8.0	5.8	**10.3**	7.3	9.8	7.5	13.0	9.5	13.5	8.8	14.5	8.5	16.8	8.5	**20.3**	7.8	**20.0**	7.5	**23.0**
P	NS		NS		0.0374		NS		NS		NS		NS		NS		0.0001		0.0079		0.0125	

Table 2.3. Raw counts of bees visiting feeders after switching from sugar water to treatment (5 ng/mL imidacloprid). Time represents how long after switching feeders to treatment (IM indicates imidacloprid) and control (S indicates sugar water control) location. A significantly greater average count is bolded. Significance values from paired t-test greater than $P > 0.05$ are indicated by NS.

	Number of bees visiting each feeder (5 ng/mL imidacloprid)																			
	1 min		2 min		3 min		4 min		5 min		6 min		7 min		8 min		9 min		10 min	
	IM	S	IM	S	IM	S	IM	S	IM	S	IM	S	IM	S	IM	S	IM	S	IM	S
Trial 1	66	62	87	75	98	76	105	78	99	80	102	76	94	69	86	58	91	66	84	59
Trial 2	21	20	19	21	27	21	24	25	29	29	34	32	33	28	29	30	31	24	31	24
Trial 3	37	29	33	24	43	26	44	37	44	48	48	51	53	61	60	65	65	66	71	69
Trial 4	33	43	36	43	29	27	37	25	37	26	38	39	35	38	39	33	39	25	42	28
Trial 5	4	13	11	13	11	14	14	15	16	14	18	17	16	14	19	15	13	11	18	12
Trial 6	11	16	15	17	17	13	20	16	15	14	19	16	16	11	15	11	17	14	17	13
Average	28.7	30.5	33.5	32.2	37.5	29.5	40.7	32.7	40.0	35.2	43.2	38.5	41.2	36.8	41.3	35.3	**42.7**	34.3	**43.8**	34.2
P	NS		NS		NS		NS		NS		NS		NS		NS		0.0442		0.0197	

Table 2.4. Raw counts of bees visiting feeders after switching from sugar water to treatment (acetamiprid). (a) Low acetamiprid concentration– 50 ng/mL (b) Middle range acetamiprid concentration - 500 ng/mL (c) High acetamiprid concentration – 5 µg/mL. Time represents how long after switching feeders to treatment (AC indicates acetamiprid) and control (S indicates sugar water control) location. No significance values from paired t-test are shown as all were greater than $P > 0.05$.

a. Number of bees on each feeder (50 ng/mL acetamiprid)

	1 min		2 min		3 min		4 min		5 min		6 min		7 min		8 min		9 min		10 min	
	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S
Trial 1	87	31	89	86	92	112	91	99	95	99	94	101	94	94	94	112	91	111	89	108
Trial 2	100	68	98	85	98	93	113	85	101	90	98	96	98	99	102	100	105	96	99	100
Trial 3	96	131	102	114	95	101	94	102	105	113	97	114	101	111	95	113	103	116	100	96
Trial 4	84	125	116	123	133	113	131	107	125	117	135	105	134	110	134	113	131	100	124	95
Average	91.8	88.8	101.3	102	104.5	104.8	107.3	98.3	106.5	104.8	106	104	106.8	103.5	106.3	109.5	107.5	105.8	103	99.8

b. Number of bees on each feeder (500 ng/g acetamiprid)

	1 min		2 min		3 min		4 min		5 min		6 min		7 min		8 min		9 min		10 min	
	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S
Trial 1	60	56	63	60	72	64	71	69	74	70	76	76	72	78	75	70	71	73	74	71
Trial 2	69	63	60	66	60	63	59	60	65	68	73	66	73	65	74	59	70	66	66	73
Trial 3	53	48	58	74	66	65	67	54	66	60	55	61	61	61	63	55	70	50	60	43
Trial 4	27	73	47	76	54	77	64	64	64	58	61	66	65	66	63	68	63	70	65	75
Average	52.3	60.0	57.0	69.0	63.0	67.3	65.3	61.8	67.3	64.0	66.3	67.3	67.8	67.5	68.8	63.0	68.5	64.8	66.3	65.5

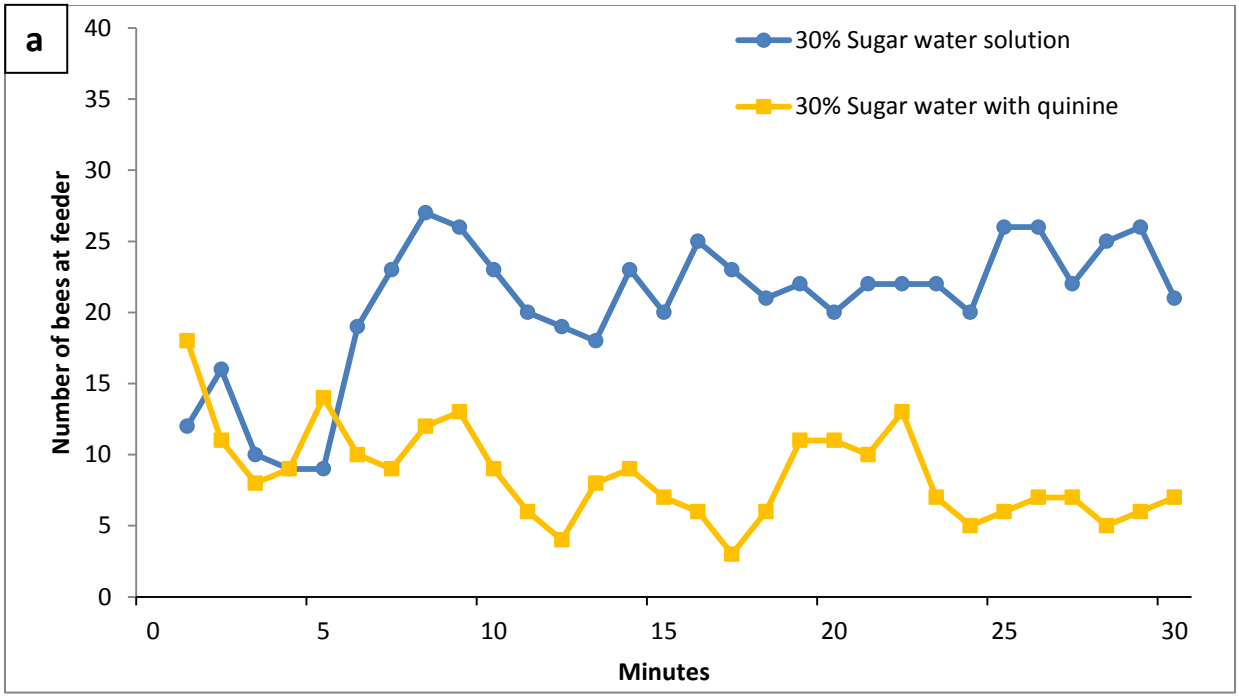
c. Number of bees on each feeder (5 µg/mL acetamiprid)

	1 min		2 min		3 min		4 min		5 min		6 min		7 min		8 min		9 min		10 min	
	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S	AC	S
Trial 1	86	73	81	92	84	89	78	100	69	93	79	81	80	90	68	96	70	105	73	104
Trial 2	39	49	65	51	70	63	72	65	71	57	72	60	68	72	71	77	72	71	72	74
Trial 3	51	33	61	43	62	49	62	48	61	46	48	42	56	52	60	54	63	55	57	52
Trial 4	51	43	58	41	66	50	70	51	60	62	76	63	71	53	67	58	69	64	59	63
Average	56.8	49.5	66.3	56.8	70.5	62.8	70.5	66.0	65.3	64.5	68.8	61.5	68.8	66.8	66.5	71.3	68.5	73.8	65.3	73.3

Figure 2.1. Free flight feeder for each feeding station. The box was made from acrylic sheeting and tubes and is accessible only through tube entrances such that bees could be seen and counted entering and exiting the feeder. The feeder was placed on a scale so that the amount consumed could be tracked live through a webcam positioned directly above for an overhead view.

Figure 2.2. Screenshot taken during feeding assay and pasted into Image J for analysis. The yellow markers show how software was used to count bees every minute (see clock in upper left corner).

Figure 2.3. Raw counts of bees visiting feeders after switching from sugar water to treatment. Trials (a) through (d) of number of bees counted at sugar water feeders with and without 15 mM quinine treatment every minute over the course of 30 minutes. These data are presented in raw table form in Table 2.2, with significant differences highlighted.



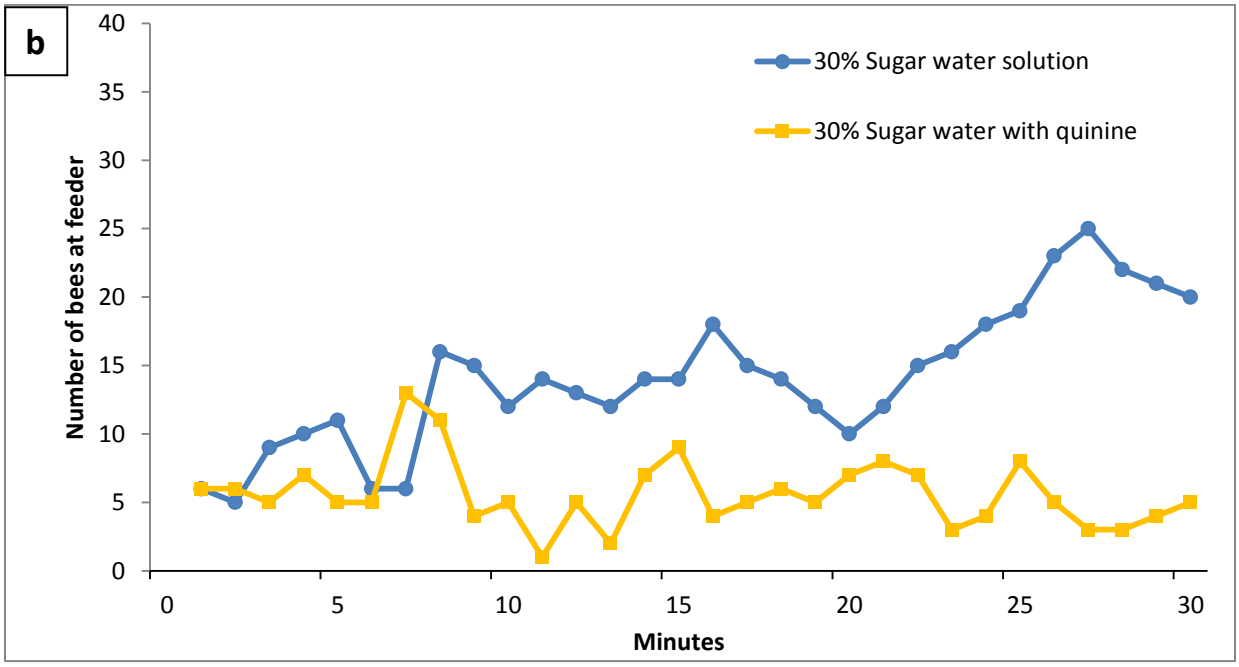
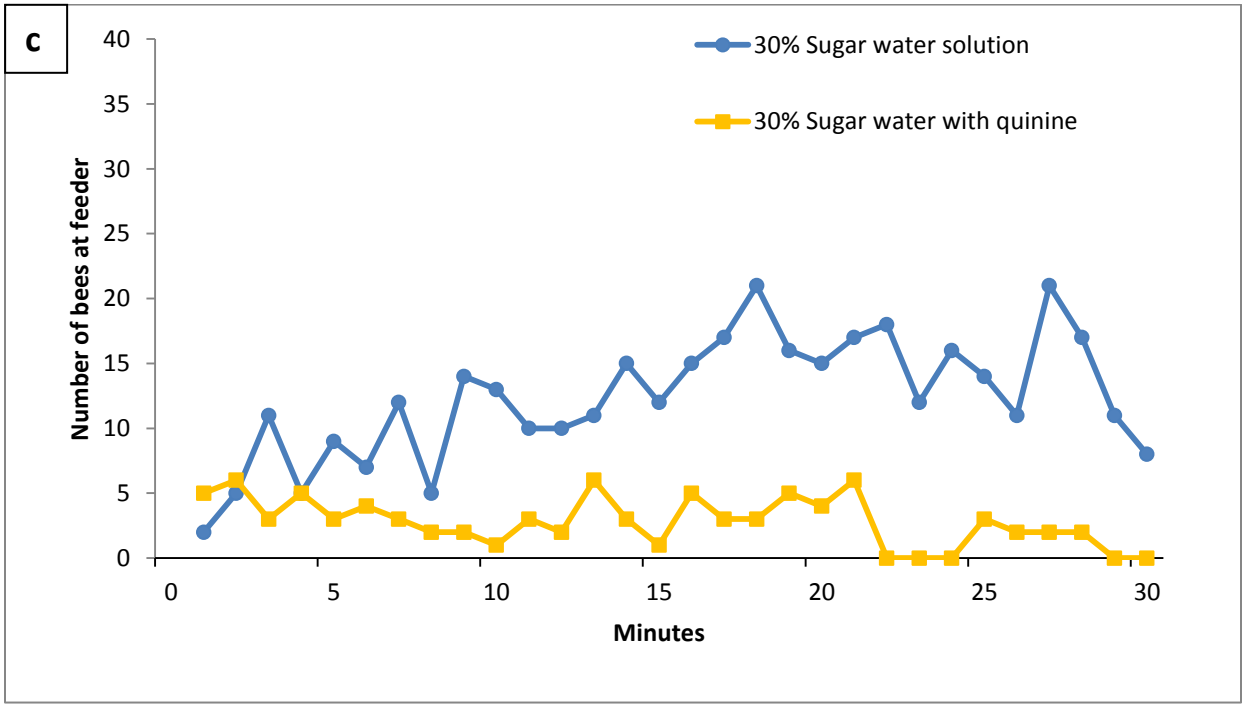


Figure 2.3 (cont'd).



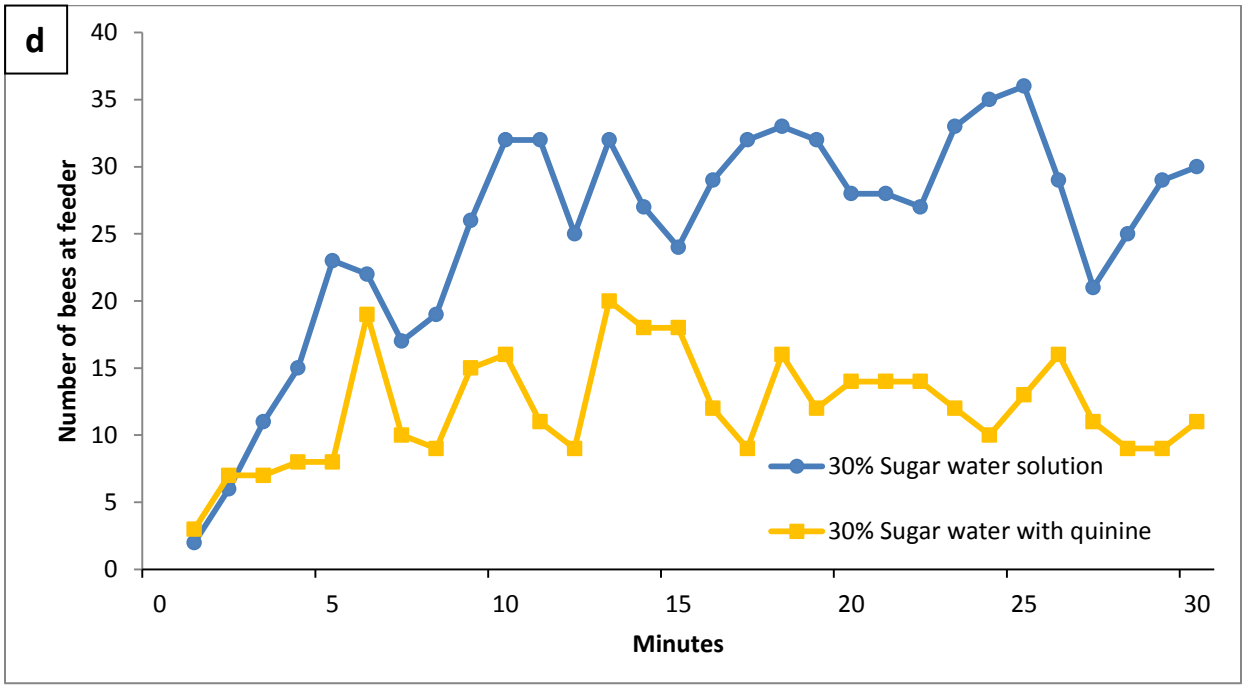
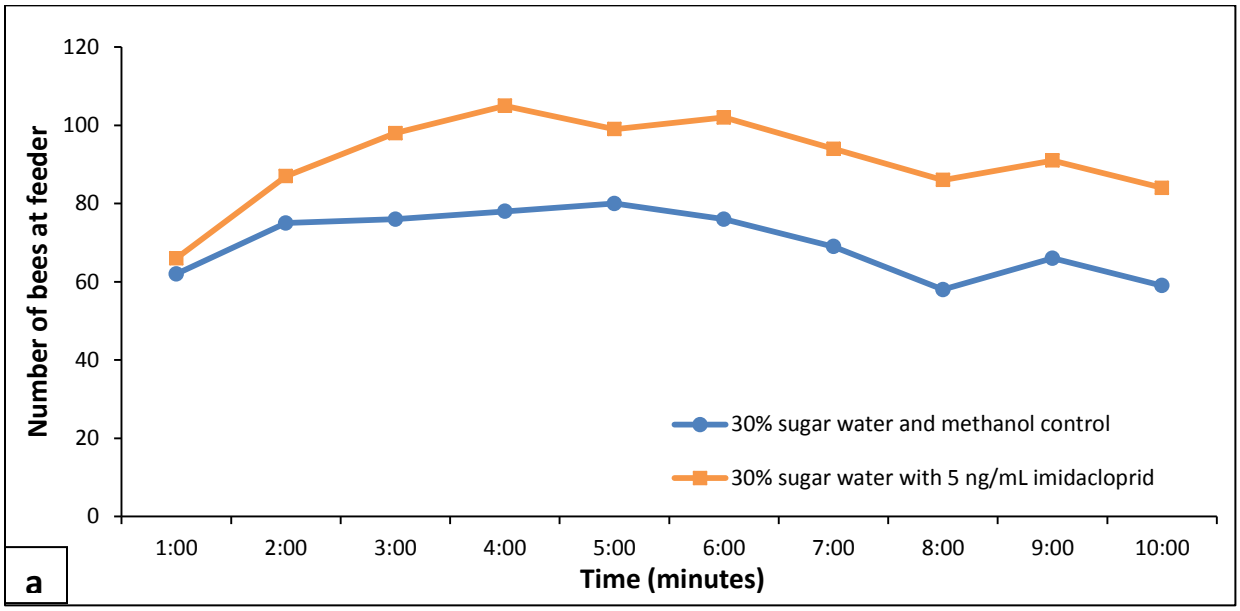


Figure 2.4. Raw counts of bees visiting feeders after switching from sugar water to treatment. The number of bees counted for the feeder with imidacloprid (5 ng/mL) and the solvent (methanol) control every minute for 10 minutes are shown on the y axis. These data are presented in raw table form in Table 2.3, with significant differences highlighted. Tandem replicates are grouped together (treatment on the west side of the arena by one hive, and on the east side of the arena by the other hive (a&b, c&d, and e&f). Note that foraging rates varied by day and hive, so number ranges may change on the y-axes.



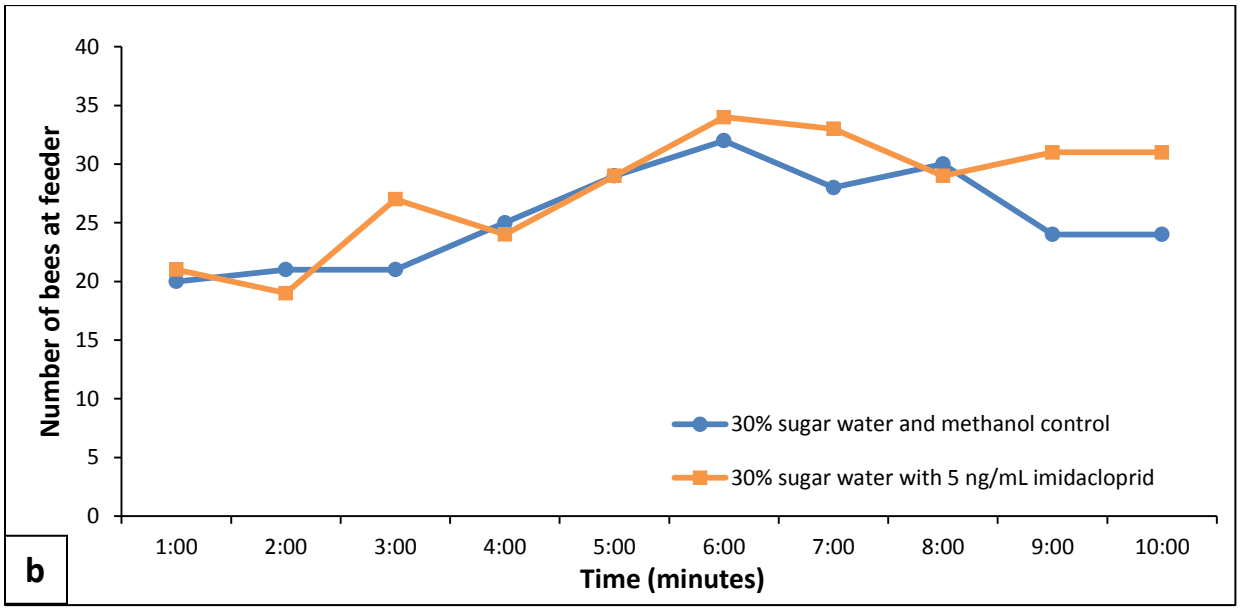
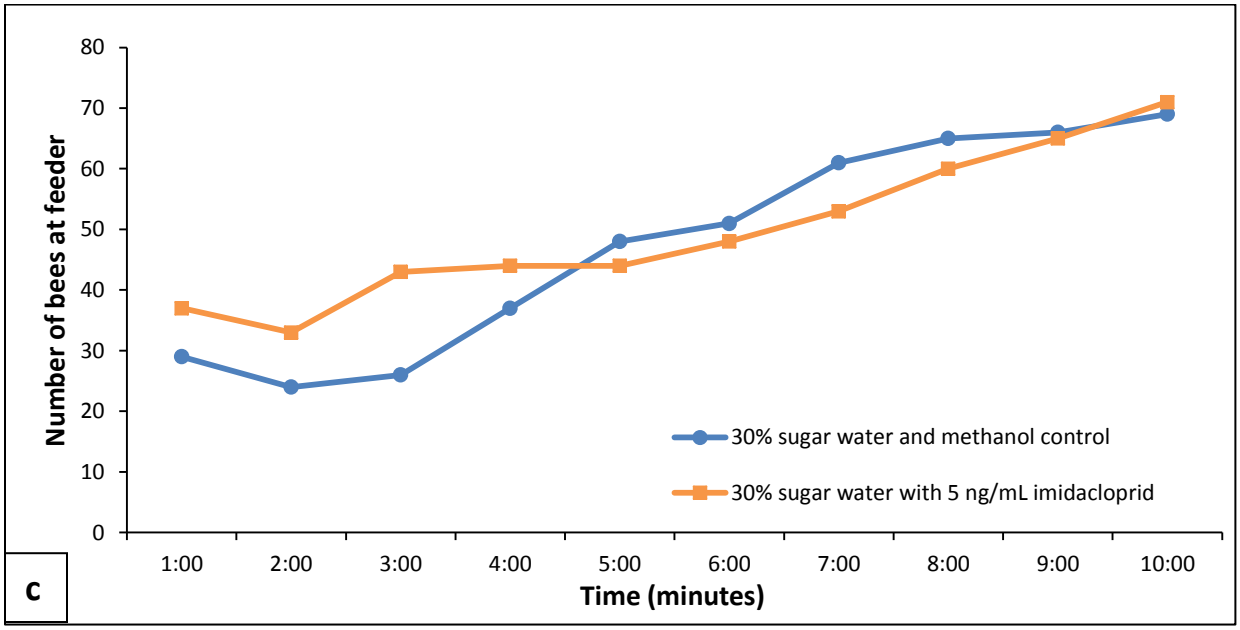


Figure 2.4 (cont'd).



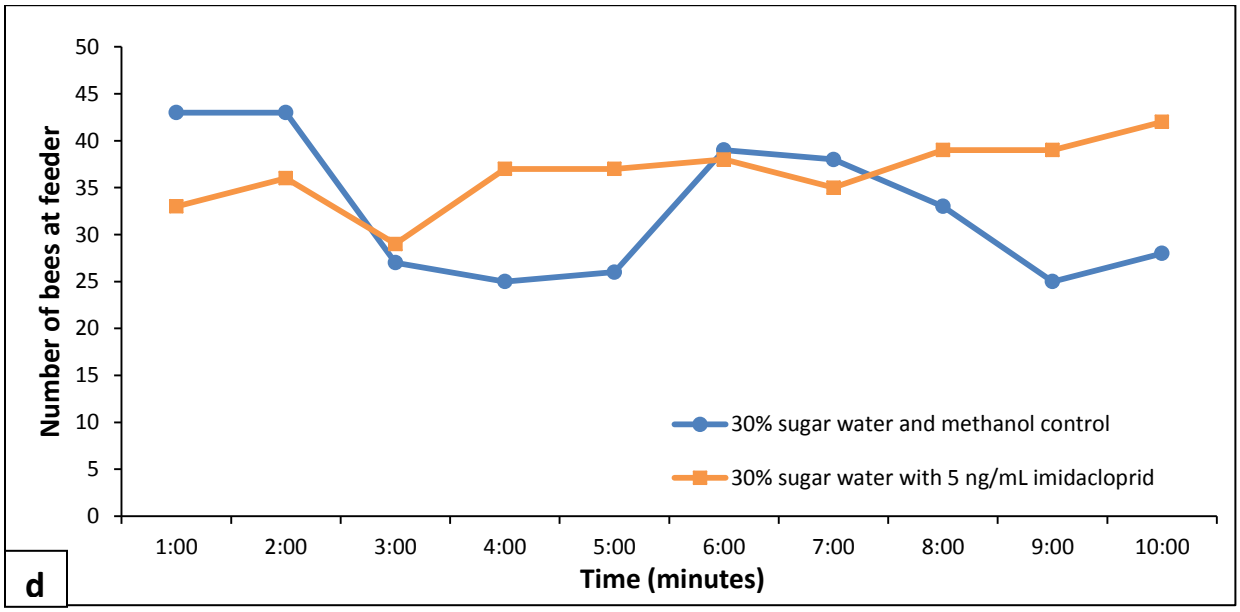
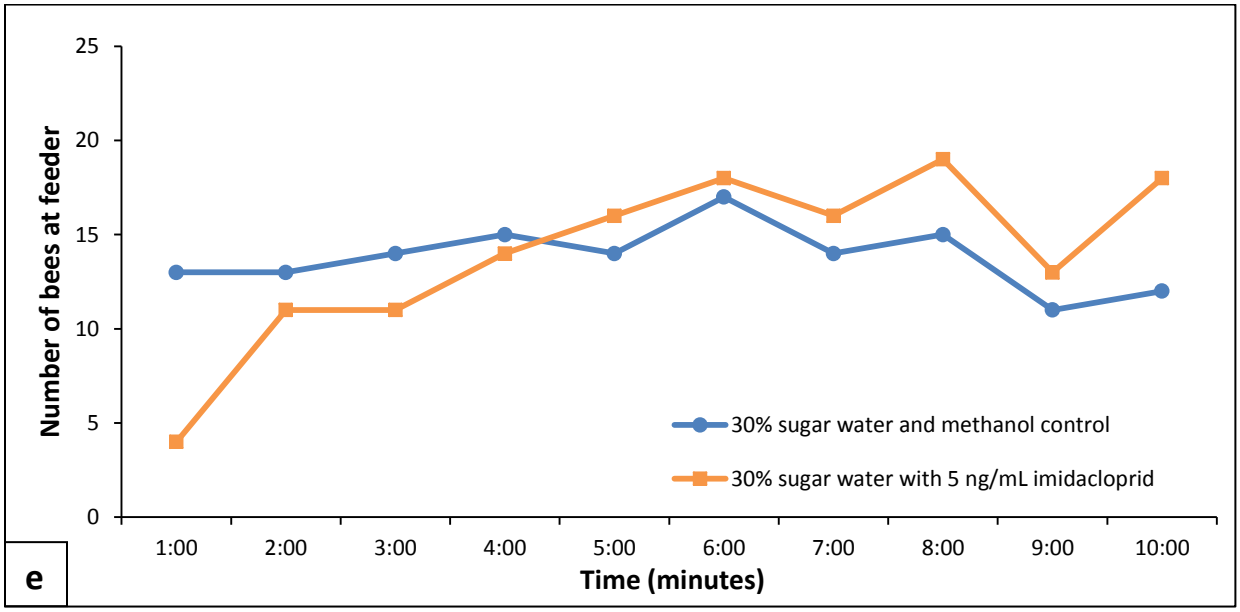


Figure 2.4 (cont'd).



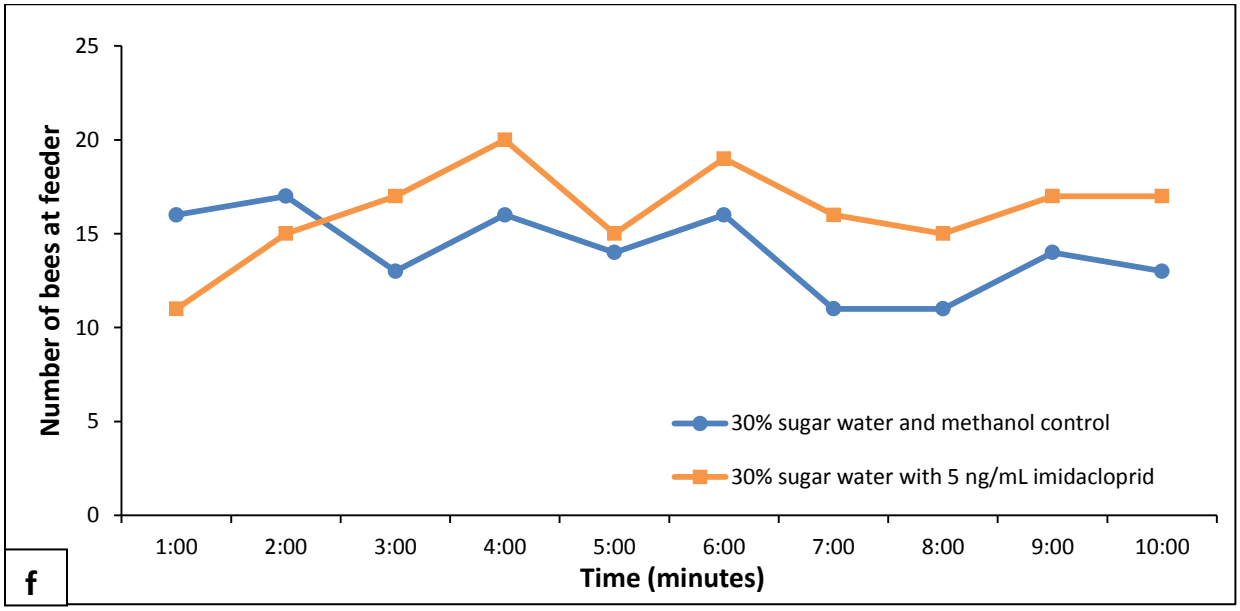
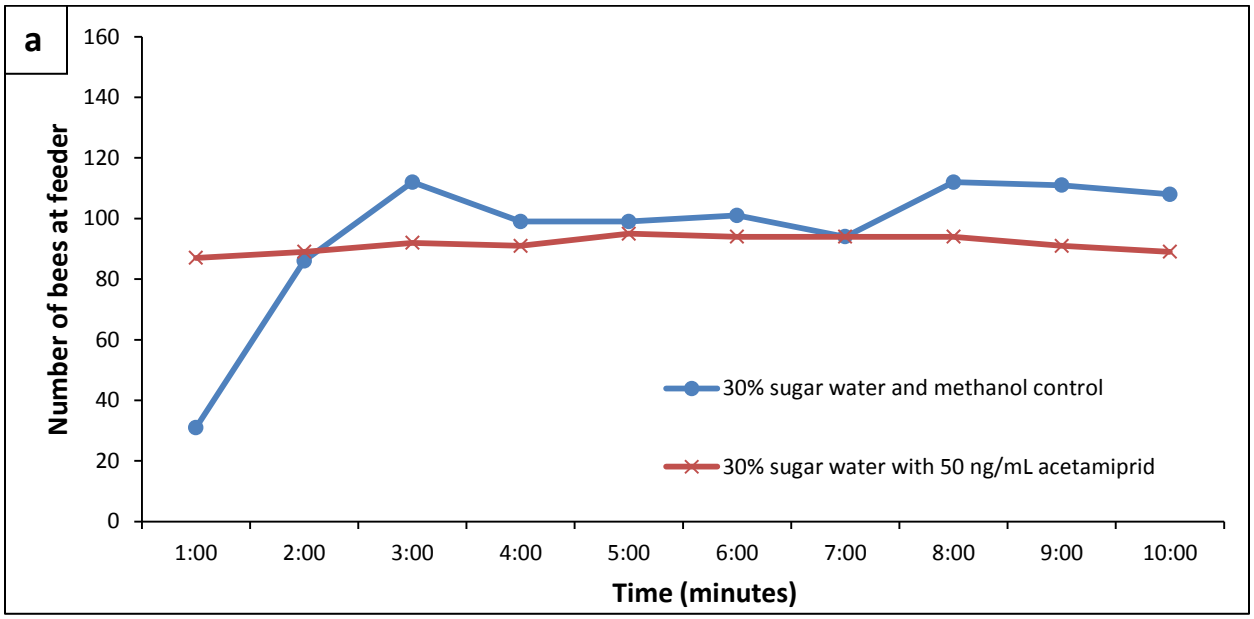


Figure 2.5. Number of bees counted at sugar water feeders with and without the lowest concentration of acetamiprid tested (50 ng/mL) every minute over the course of 10 minutes. Trials a and b (as well as c and d) were conducted with separate hives in tandem, with the treatment on opposite sides of the arena (East and West) to minimize effects of sunlight on foraging rates.



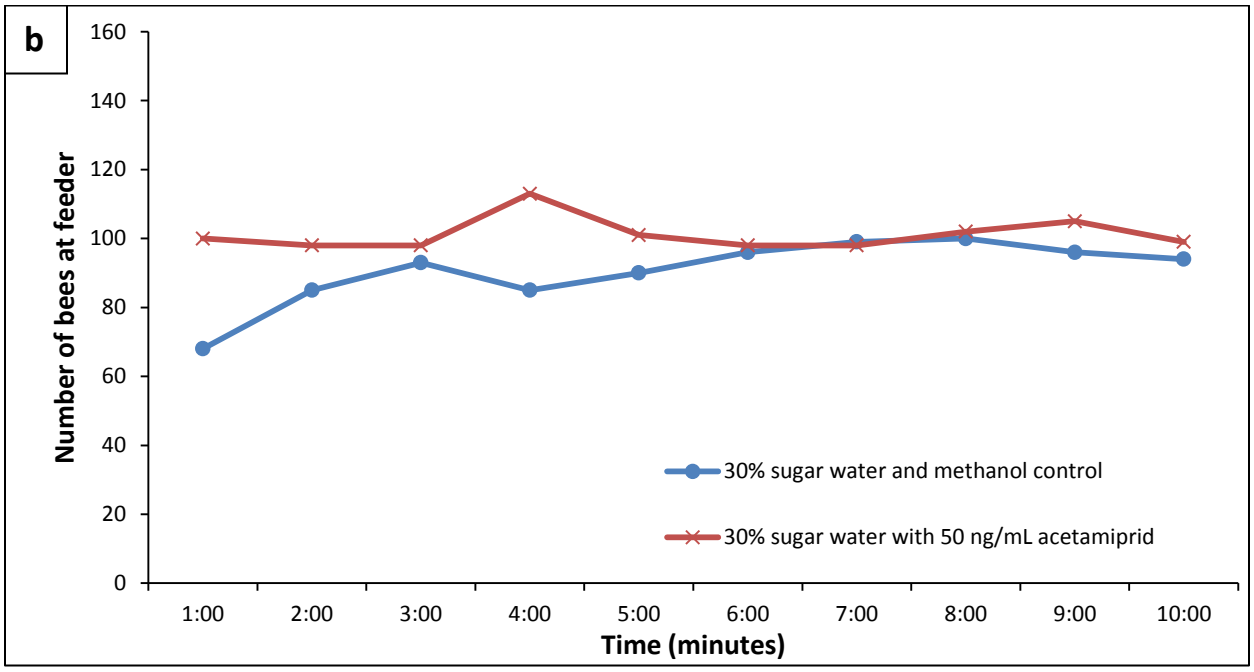


Figure 2.5 (cont'd).

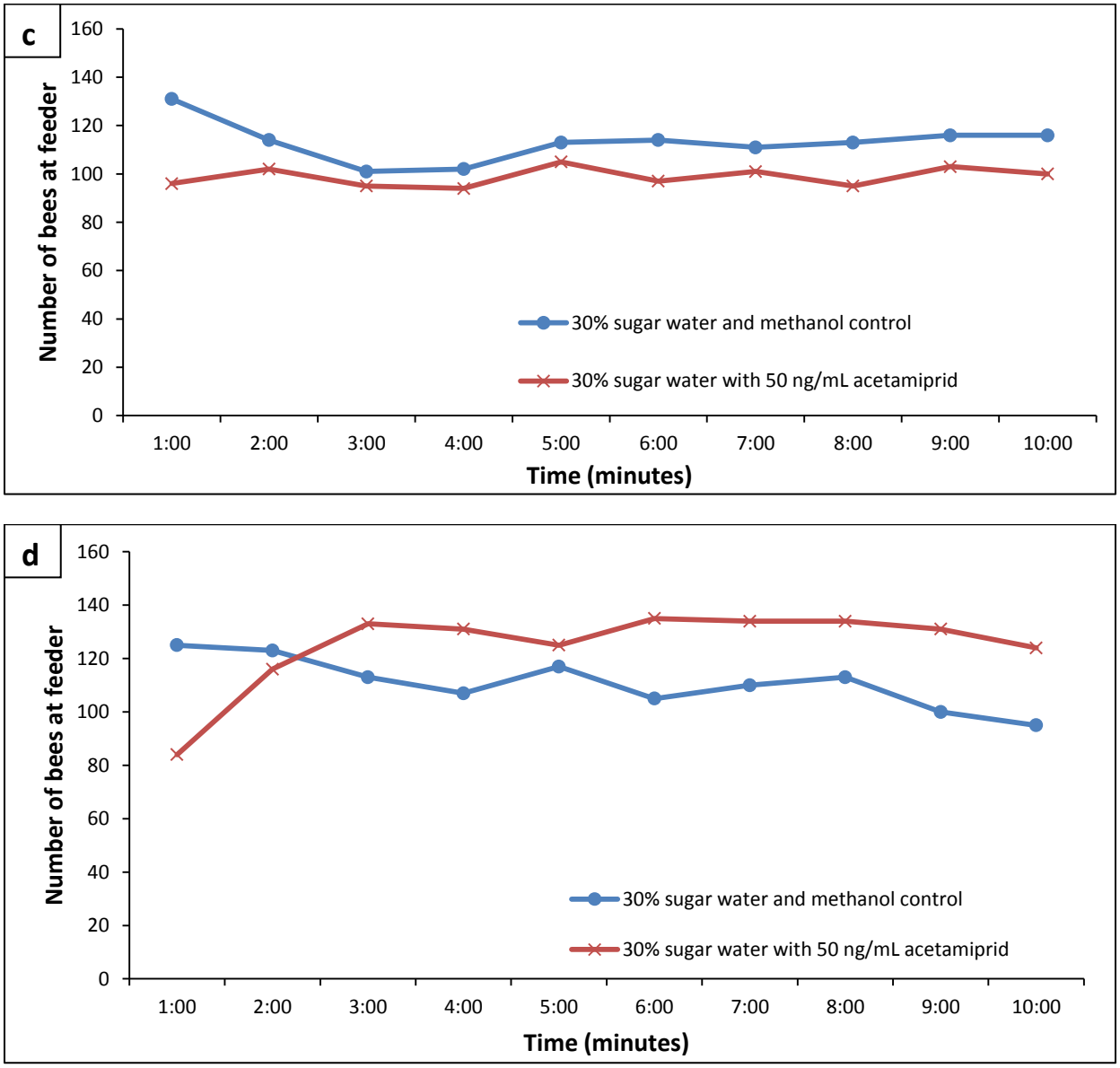


Figure 2.6. Number of bees counted at sugar water feeders with and without mid-range concentration acetamiprid (500 ng/mL) every minute over the course of 10 minutes. Trials a and b (as well as c and d) were carried out with separate hives in tandem, with the treatment on opposite sides of the arena (East and West) to minimize effects of sunlight on foraging rates.

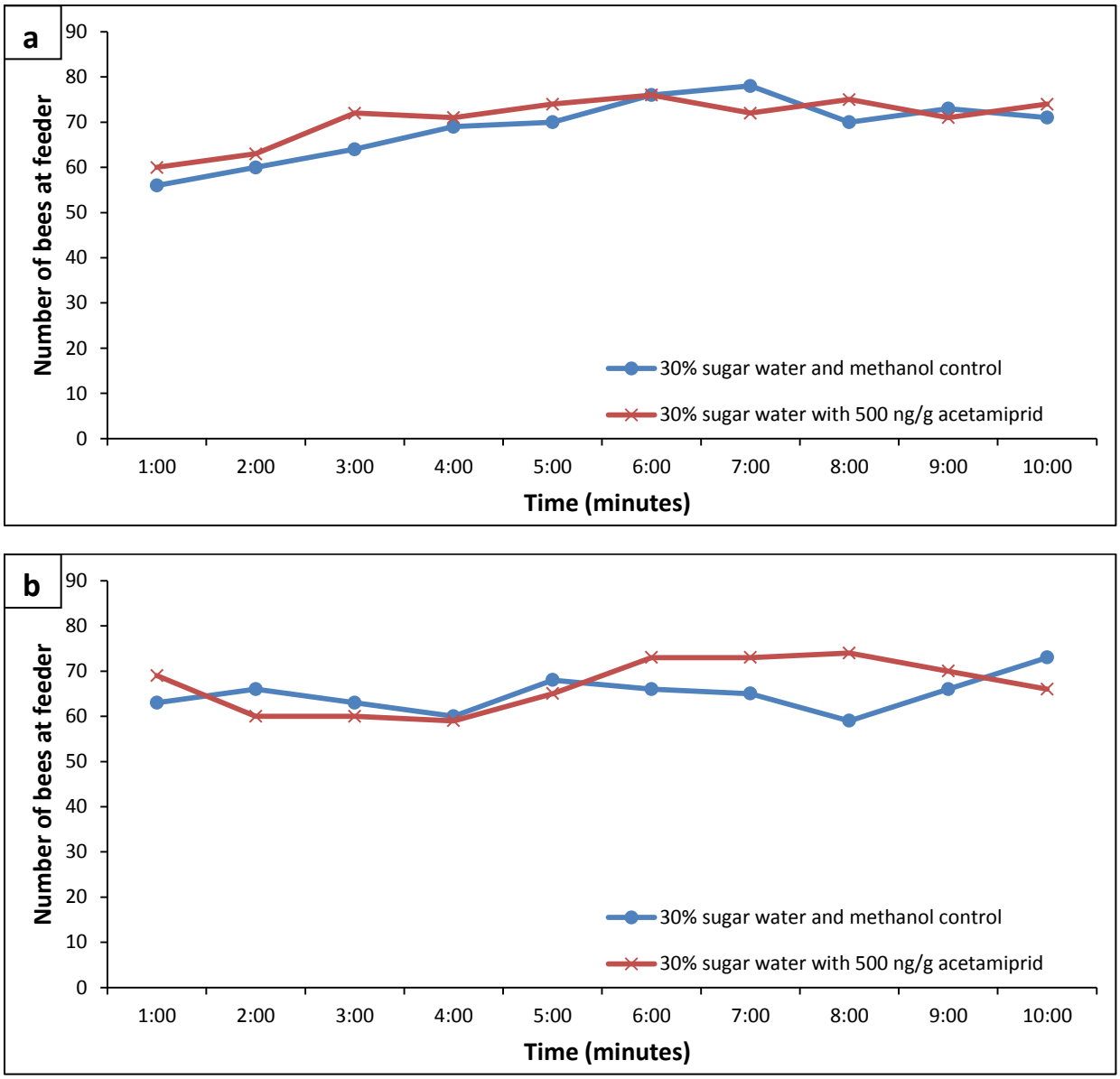
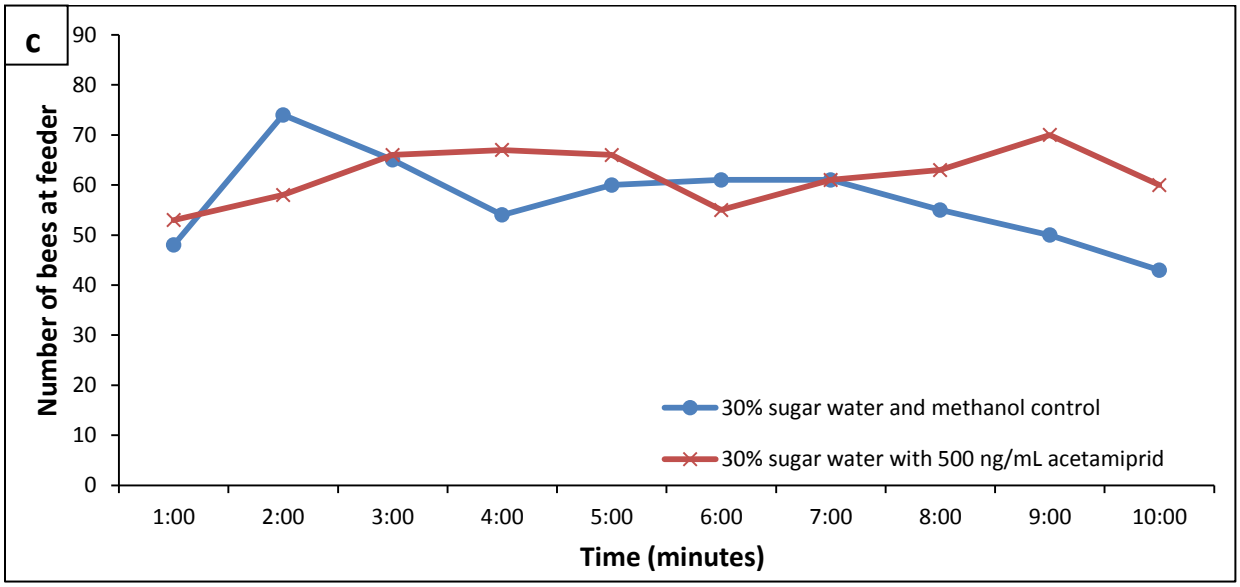


Figure 2.6 (cont'd).



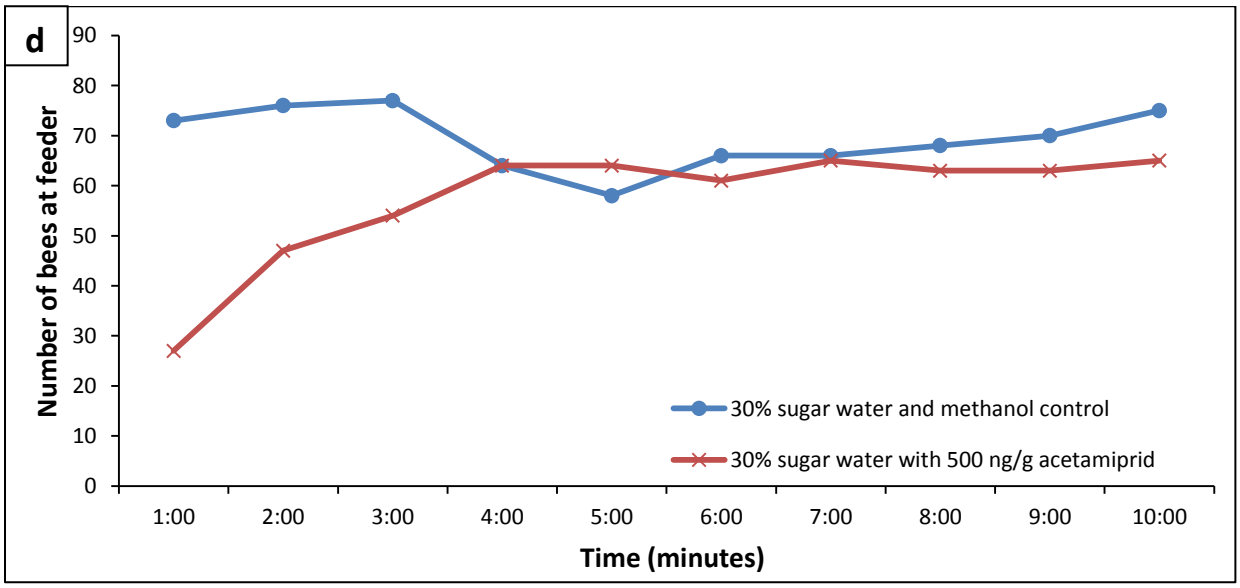


Figure 2.7. Number of bees counted at sugar water feeders with and without highest concentration of acetamiprid tested (5 µg/mL) every minute over the course of 10 minutes. Trials a and b (as well as c and d) were run with separate hives in tandem, with the treatment on opposite sides of the arena (East and West) to minimize effects of sunlight on foraging rates. Note that foraging rates did vary by day and hive, so number ranges may change on the y axes.

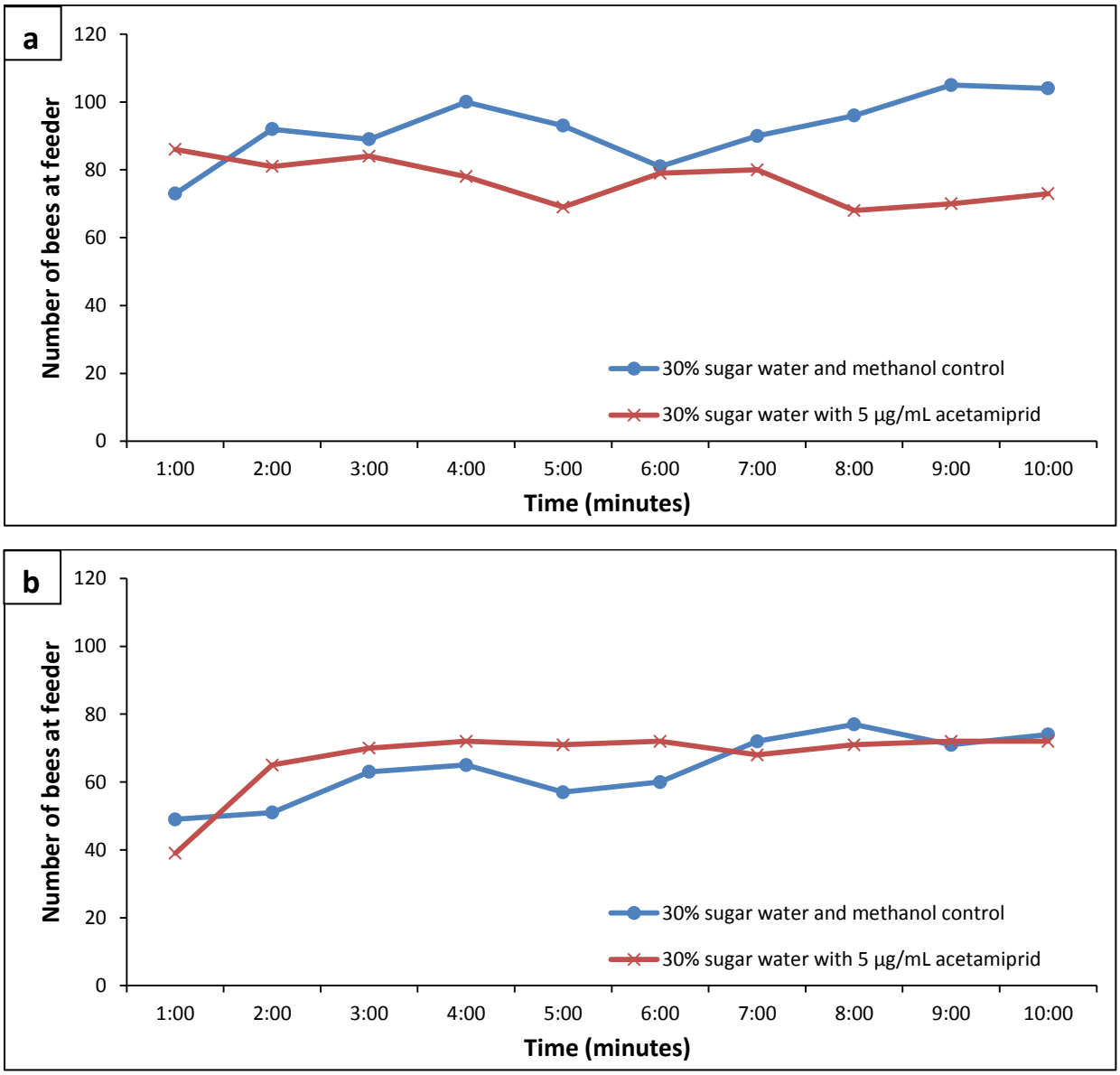
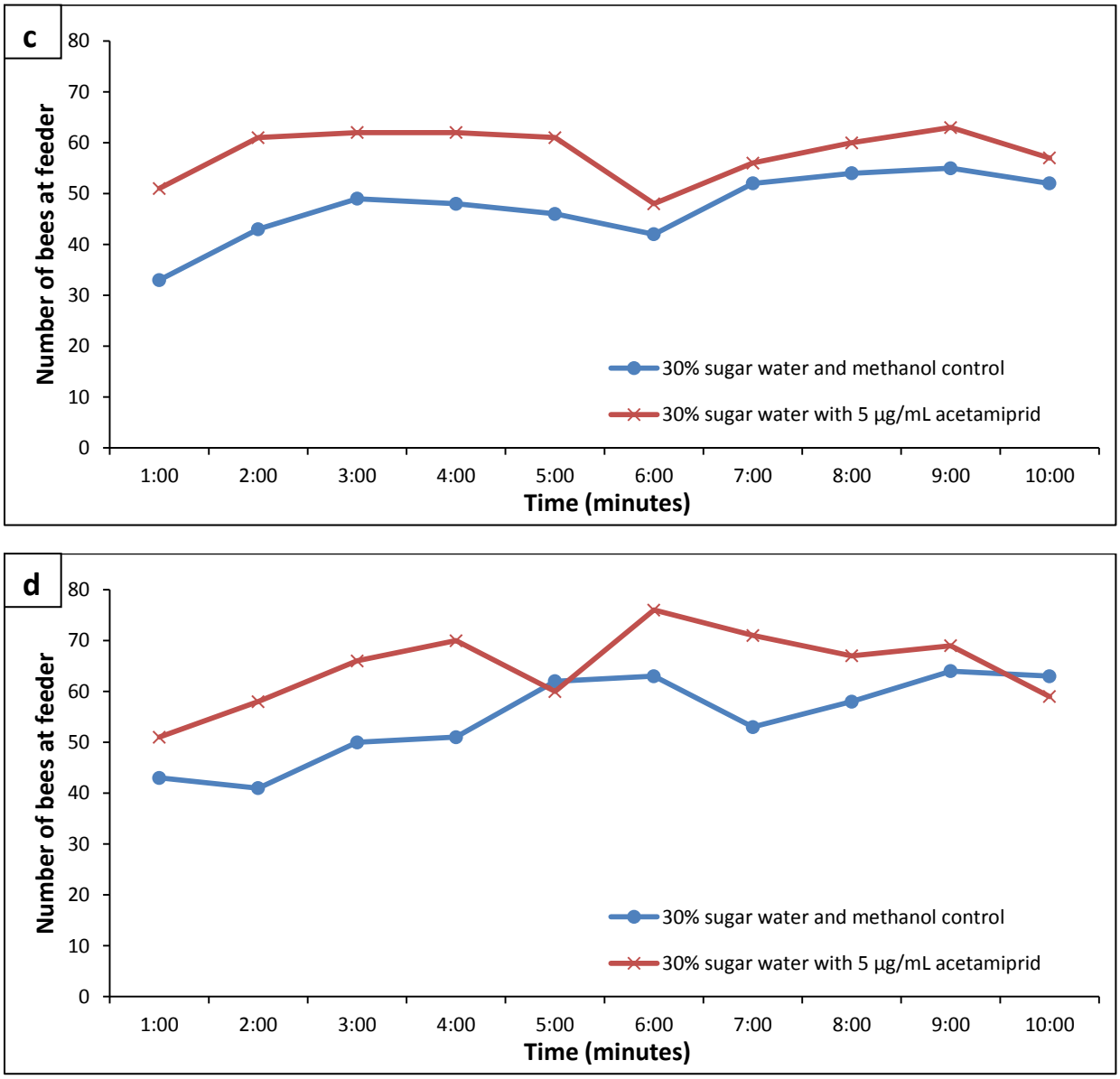


Figure 2.7 (cont'd).



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